Expression and Characterization of E-LecEGF for Structural Study and Assay Development

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Abbreviations

Ab	Antibody
ABTS	2, 2'-azino-di-[3-ethylbenzthiazoline-6-sulfonic acid]
AcNPV	Autographa californica nuclear polyhedrosis virus
Amp	Ampicilline
AP	Alkaline phosphatase
APS	Ammonium persulfate
Arg	L-Arginine
Asn	L-Asparagine
Asp	L-Aspartic acid
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
Blast	Basic local alignment search tool
BmPV	Bombyx mori polyhedrosis virus
BSA	Bovine serum albumine
C	Constant region
CAM	Chloramphenicol
cAMP	Cyclic adenosine monophosphate
CD	Consensus repeat domains
CD34	Cell differentiation antigen 34, sialomucin
cDNA	Complementary deoxyribonucleic acid
CDR	Complementarity determining region
CH ; CL	Constant region of heavy chain; constant region of light chain
CH_3CN	Acetonitrile
CHO	Chinese hamster ovary
CHO-E-sel/IgG	Chinese hamster ovary expressing the extracellular domains of human E-selectin fused with a C-terminal human IgG1 tag
COS	African green monkey's kidney cells
CRD	Carbohydrate recognition domain
Cys	Cystein
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
dNTP	Deoxynucleotidetriphosphate
DPBS	Dulbeccco's PBS
dsDNA	Double strand DNA
DTT	Dithiothreitol
E.coli	Escherichia coli
EC50	Concentration for half maximal effect
EDTA	Ethylendiaminotetraacetic acid
EGF	Epidermal growth factor

E-LecEGF	E-selectin lectin and EGF-like domains
ELISA	Enzyme linked sorbent assay
ESI-MS	Electro-spray ionization mass spectroscopy
Fab	Fragment antigen binding
FBS	Fetal bovine serum
Fc region	Fragment crystallizable region
FCS	Fetal calf serum
FPLC	Fast performance liquid chromatography
Fuc	Fucose
Fuc T	Fucosyl transferase
Gal	Galactose
Gal*N*Ac	*N*-Acetyl-D-galactosamine
GAM	Goat anti-mouse
Glc	D-Glucose
Glc*N*Ac	*N*-Acetyl-D-glucosamine
Gln	L-Glutamine
GlyCAM	Glycosylation dependent cell adhesion molecule
H	Heavy chain
HAB	Hepes assay buffer
HAB20	HAB buffer containing 20mM $CaCl_2$
*h*E-LecEGF	The lectin and EGF-like domains of human E-selectin
*h*E-LecEGF/IgG	*h*E-LecEGF fused with a C-terminal *h*IgG1 tag
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
hE-selectin/IgG	The lectin, EGF-like and CR domains of human E-selectin fused with a C-terminal human IgG1 tag
HEV	High endothelial venules
*h*IgG1 tag	the partial CH_1 domain and complete CH_2 and CH_3 domains of human IgG1 sequence
Hi-5	High Five^{TM} insect cells
His	Histidine
HL-60	Human promyelocytic leukemia cells
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HT	Hypoxanthine
HTS	High throughput screening
HUVECs	Human umbilical vein endothelial cells
IC50	Concentration for half maximal inhibition
ICAM	Intracellular adhesion molecule
ICAM-1	Intercellular cell adhesion molecule 1
IFA	Incomplete Freund's adjuvant
IFN	Interferon
IgG1	Immunoglobulin G1
IL ; hIL	Interleukin ; human interleukin
IMAC	Immobilized metal ion affinity chromatography

IMDM	Iscove's modified Dulbecco's medium
IPTG	Isopropyl-β-D-Thiogalactosid
IMP	Institute of Molecular Pharmacy, University of Basel
K	Lysine
Kan	Kanamycine
KD	Equilibrium binding affinity
Kd	Dissociation constant
kDa	Kilo Dalton
KOAc	Potassium acetate
KPBS	Kreis' PBS
L	Light chain
LAD	Leukocyte adhesion deficiency
LB	Lauria Bretani
LecEGFFlag	E-selectin lectin and EGF-like domains with a C-terminal Flag epitope
LFA	Leukocyte function associated antigen
LPS	Lipopolysaccharide
M.O.I.	Multiplicity of infection
mAb	monoclonal antibody
MadCAM	Mucosal addressin cell adhesion molecule
Man	Mannose
MAPK	Mitogen-activated protein kinase
MBP	Mannose binding protein
M-CSF	Macrophage colony-stimulating factor
MECA	multi-endocrine cellular antigen
MEM	Minimal essential medium
min	Minute(s)
MS	Mass spectrometry
N	Asparagine
NaOAc	Sodium acetate
NBT/BCIP	Nitroblue tetrazoliumchloride 5-bromo-4-chloro-3-indolylphosphate
Neu*N*Ac	*N*-acetylneuraminic acid
NF-kB	Nuclear factor kappa B
NK	Neutral killer
NMR	Nuclear magnetic resonance
OD	Optical density
OPD	o-phenylenediamine dihydrochloride
PAGE	Polyacrylamide gel-electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PECAM	Platelet endothelial cell adhesion molecule
PEG	Polyethylene glycol
Pfu	Plaque forming units
P-LecEGF	The lectin and EGF-like domains of P-selectin
PMSF	Phenylmethane sulfonylfluoride

PNGaseF	*N*-glycosidase F
POD	Peroxidase
PSGL-1	P-selectin glycoprotein ligand-1
Q	Glutamine
R	Arginine
R^2	Correlation coefficient
Rcf	Relative centrifugal force
RP-HPLC	Reversed phase high pressure liquid chromatography
Rpm	Rounds per minute
RT	Room temperature
SDS	Sodiumdodecylsulfate
SDS-PAGE	Sodium-dodecyl-sulfat polyacrylamide-gelelectrophoresis
Sf9	Spodoptera frugiperda 9 cells
SFM	Serum-free medium
Sia	Sialic acid
sLe^a	Sialyl Lewis A
sLe^x	Sialyl Lewis X
SLE sequence	The signal, lectin and EGF-like domains of human E-selectin
SN	Supernatant
SPR	Surface plasmon resonance
STD	Saturation transfer difference
TB	Terrific broth
TBS	Tris buffered saline
TEMED	tetramethylethylenediamine
Tet	Tetracycline
TFA	Trifluoroacetic acid
T_m	Melting temperature
TNF	Tumor necrosis factor
Tris	(Hydroxymethyl)-Aminomethan
TTBS	TBS with 0.05% Tween 20
V	Variable region
VCAM-1	Vascular cell adhesion molecule
V_H ; V_L	Variable region of heavy chain ; variable region of light chain
Y	Tyrosine

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Summary

Human E-selectin (*h*E-selectin) is a cell adhesion molecule expressed on the membrane of endothelial cells. It is a C-type lectin whose key role is to mediate the initial rolling and adhering of leukocytes in the leukocyte recruitment in inflammation and metastasis of some cancer cells. It is fundamentally involved in many physiological and pathological processes, and hence is an attractive target for developing anti-inflammation drugs. The lectin and EGF domains of *h*E-selectin (*h*E-LecEGF) were identified as the minimum functional unit. Crystal structures of *h*E-LecEGF complexed with its natural ligand, tetrasaccharide sialyl Lewis^x (sLe^x), as well as NMR studies of *h*E-selectin/IgG bound with this ligand, have been reported and utilized as the structural basis for the development of potent antagonists for *h*E-selectin. More potent antagonists with better binding affinity than sLe^x, such as CGP69669, were reported, but their binding modes in *h*E-LecEGF remain unknown. To obtain the improved structural information of *h*E-LecEGF complexed with an antagonist and develop more potent antagonists of *h*E-selectin are challenging tasks. To meet the demands of the protein for the structure determination and the binding assay, a sufficient amount of pure and active *h*E-LecEGF is needed.

In this thesis, insect cell expression systems were initially used to produce the *h*E-LecEGF protein. *h*E-LecEGF was cloned, transiently expressed and characterized in Sf9 and High five™ cells. The expression plasmid pFastBacYJSE was constructed for expression of the *h*E-LecEGF protein fused with a N-terminal Flag tag. The recombinant baculovirus was generated and used in the expression of protein in the suspension culture. Pure *h*E-LecEGF was obtained by anti-Flag M2 affinity chromatography under the optimized condition. The purified protein was active and glycosylated, as identified by mAb 7A9 and glycan detection, respectively. Unfortunately, the homogeneous *h*E-LecEGF protein was not obtained after the deglycosylation with PNGase F and N-glycosidase A.

*h*E-LecEGF was then cloned, stably expressed and characterized in CHO K1 cells. Stable subclones CHO-YJES and CHO-YJEGS expressing the *h*E-LecEGF protein with or without a human IgG1 tag were achieved. The CHO-YJES construct was used for production. The monoclonal anti-E-selectin functional blocking antibody 7A9 (mAb 7A9) was produced, purified and coupled to sepharose for functional purification of the *h*E-LecEGF protein. Highly pure *h*E-LecEGF protein was obtained in a one-step purification with an mAb 7A9 coupled column. Page, western-blotting, ELISA, MS and NMR were performed to characterize the *h*E-LecEGF protein. Pure, monomeric and active *h*E-LecEGF with the molecular weight of

20.444 kDa was obtained. In contrast to the insect cell expression system, pure, active and uniform deglycosylated *h*E-LecEGF protein was obtained after treatment with PNGase F and purification by a Sepharose Q matrix. A prescreening of the crystallization condition of *h*E-LecEGF was also performed using a sitting-drop method.

Furthermore, a novel cell-free assay “capture-binding assay” was developed with the tag-free *h*E-LecEGF protein to evaluate the binding activity of the *h*E-LecEGF protein and the binding affinity of *h*E-selectin antagonists. The rIC_{50} of six *h*E-selectin antagonists was determined. The obtained results were in close agreement with the published results. Compared to the previously unstable polymer assay with *h*E-selectin/IgG, the capture-binding assay with *h*E-LecEGF is accurate, sensitive and reproducible. It can correctly evaluate the binding affinities of *h*E-selectin antagonists. In addition, the antibody BBA1 was used to solve the problem of immobilization of the *h*E-LecEGF protein on ELISA plates in the assay.

1. Introduction

1.1 Selectins

Lectins are carbohydrate-binding proteins. They typically play a role in biological recognition phenomena involving cells and proteins. Lectins are not enzymes. They bind to soluble carbohydrates or carbohydrate moieties of glycoproteins or glycolipids. Most animal lectins can be classified as either intracellular lectins or extracellular lectins (Table 1.0).

Table 1.0. Intracellular lectins and extracellular lectins.

Intracellular lectins	Extracellular lectin
● L-type lectins (β-sandwich)[1,2]	● C-type lectins (unique α/β)[3,4]
	-Selectins[5,6] -DC-Sign[7] -Asialoglycoprotein receptor[8] -Dectins[9,10] -Mannose-binding protein[11,12] -etc.
● P-type lectins (unique β-rich)[13]	● I-type lectins[14] (Siglecs[15], Ig superfamily[16])
● Calnexin[17]	● R-type lectins (β-trefoil)[18]
	● Galectins (β-sandwich)[19]

Cellular adhesion is the binding of a cell to another cell, a surface or a matrix. Cell adhesion molecules (CAMs) specifically regulate cellular adhesion by interacting with molecules on the opposing cell or surface. Such cell adhesion molecules are also termed "receptors" and the molecules they recognize are termed "ligands" (or "counterreceptors").

Selectins are a family of CAMs[20,21], consisting of E-selectin, P-selectin and L-selectin. They are single-chain transmembrane glycoproteins. Since they recognize specific carbohydrate moities in a calcium-dependent manner[6], they are called C-type lectins. Selectins play an important role in the initial step of inflammation by mediating the rolling and adhering of leukocytes[22-24]. Hence, selectins are an attractive target for developing therapeutics for acute and chronic inflammatory diseases.

1.1.1 Structure of selectins

The three members of the selectin family (E-, P- and L-selectin) share common structural motifs, including a N-terminal C-type lectin domain, an epidermal growth factor (EGF)-like domain, a variable number of consensus repeats domains (CR domains), a membrane spanning segment and a short cytoplasmic region[25] (Figure 1.1).

Human E-, P- and L-selectin showed an overall homology of approximately 40% at the nucleic acid and 60% at the protein level, with the highest degree of conservation in the lectin and EGF-like domains. The sequence conservation between species of a selectin displays 60-80% homology. This high degree of conservation supports their general role in the interaction with carbohydrate determinants.

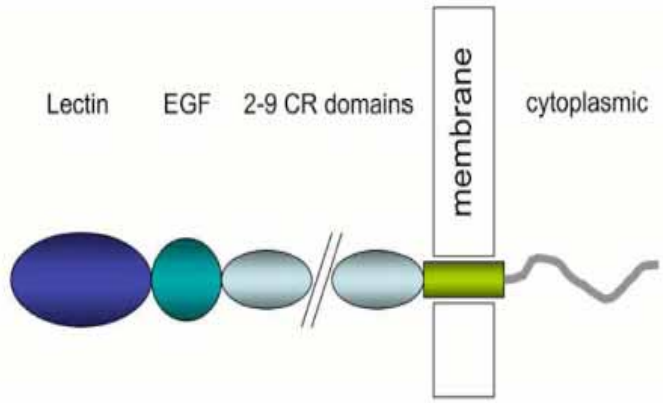


Figure 1.1. The common structure of the selectin family[26].

1.1.2 Functions of the domains in the selectin structure

The lectin domain

The N-terminal lectin domain (carbohydrate recognition domain, CRD), 120-130 amino acids in length, shows typical features of C-type animal lectins[25,27]. It plays a major role in ligand recognition and determines the carbohydrate binding specificity.

The major role of the ligand recognition by the lectin domain was identified by epitope mapping of blocking antibodies[25], blocking peptides[28,29] and site-specific mutagenesis of the lectin domain[25,30]. Five critical amino acids, namely Tyr48, Asn82, Tyr94, Lys111 and Lys113, all part of the lectin domain, were identified. These amino acids are crucial for ligand recognition. Information from the crystal structures of human E-selectin and human P-selectin co-crystallized with sialyl Lewis x (sLe^x) confirmed these findings[30,31]. The chimera proteins

generated by domain replacement of PE-1 (the E-selectin lectin domain replaced with the lectin domain from P-selectin)[32,33] and LP (the lectin and EGF-like domains of L-selectin substituted into P-selectin)[32,33] also showed that the lectin domain determines the carbohydrate binding specificity of the selectins.

The EGF-like domain

The EGF-like domain (35-40 amino acids) adjacent to the lectin domain contains six conserved cysteine residues, which form three intramolecular disulfide bonds. Compared to the lectin domain, the functional role of the EGF-like domain is less understood. It may stabilize the conformation of the lectin domain or directly interact with the ligand. It has been reported that the minimum functional unit of E-selectin consists of both the lectin and the EGF-like domain[34-36]. The chimeric selectin, containing the lectin domain of L-selectin and EGF-like domain of P-selectin, exhibited the adhesive properties of both L- and P- selectin[35], indicating that the EGF-like domain of P-selectin may participate directly in the physiological glycoprotein ligand recognition via protein-protein interaction.

The CR domains

The number of the CR domains varies among the selectin family members. Human E-, P- and L- selectin contain the six, nine and two elements, respectively. Each CR domain is about sixty amino acids in length and contains three disulfide bonds. The number of CR domains differs between species as well. Compared with human E-selectin, rat and rabbit E-selectins only have five elements. Some reports indicate that the CR domains of selectins are not required for the ligand recognition, but they may enhance the ligand binding affinity[36,37].

The cytoplasmic domain

The cytoplasmic domain of the three selectins contains 20-35 amino acids. There is no sequence homology between the different members, but it is well conserved between the different species[38], suggesting that this region has distinct functions. It probably plays a functional role in the signal transduction, but is not yet fully understood[39]. Combined with the transmembrane region, the cytoplasmic domain is responsible for the targeting of different compartments, e.g. leading P-selectin to the secretory granules, E-selectin to the plasma membrane or L-selectin to the tips of microfold on leukocytes.

1.1.3 The role of selectins in inflammation and human diseases

Inflammation is a complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells or irritants. It is a protective attempt by the organism to remove the

injurious stimuli and initiate the healing process. Inflammation is divided into acute and chronic inflammation. Acute inflammation is the initial response of the body to detrimental stimuli and is achieved by the increased movement of plasma and leukocytes from the blood into the injured tissues. Chronic inflammation causes a progressive shift in the type of cells which are present at the site of inflammation and is characterized by simultaneous destruction and healing of the inflamed tissue.

1.1.3.1 Organ specificity of leukocyte recruitment

Recruitment of leukocytes from the bloodstream into inflamed tissues is the key feature of inflammation. The leukocytes interact with endothelial cells in order to exit the blood vessel. This interaction was noted more than a century ago[40]. Only in the last two decades, however, has the mechanism been identified at a molecular level. The recruitment of leukocytes shows organ-specificity, and is either selectin-dependent or selectin-independent[40]. In general, most tissues show selectin-dependent leukocyte recruitment, including skin and mucosal membranes, kidney, skeletal muscle and heart, but not liver, lung after ischemia-reperfusion or brain[41,42].

1.1.3.2 The inflammatory cascade and its cellular mechanisms

The recruitment of leukocytes is dependent on many adhesion molecules (selectins, selectin ligands, integrins and members of the immunoglobulin superfamily), chemotactic mediators, and regulatory and activating signals in and between the cells.

The recruitment is also referred as the inflammatory cascade (Figure 1.2). It contains five major steps: (1) inflammatory stimulus, (2) tethering and rolling, (3) integrin activation, (4) firm adhesion and (5) transendothelial migration. Selectins participate in the second step, i.e. tethering and rolling.

(1) Inflammatory stimulus

The stimuli, such as proinflammatory mediators (cytokines, histamine and free radicals)[43,44], initiate the inflammatory cascade. These stimuli lead to the expression of E- and P-selectin on vascular endothelial cells.

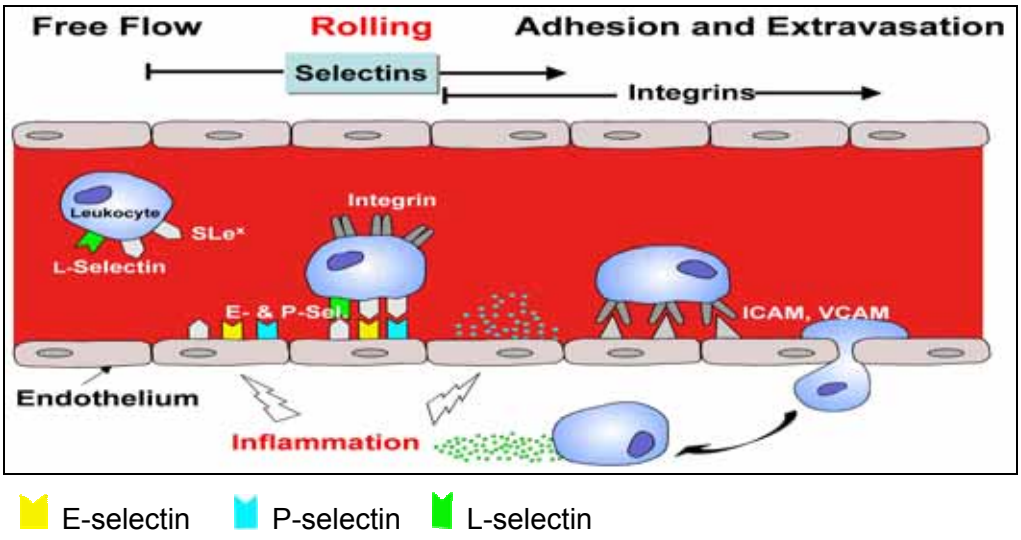


Figure 1.2. Schematic representation of the inflammatory cascade.

(2) Tethering and rolling

After cell activation by stimuli, P-selectin stored in α-granules of platelets and Waibel-Palade bodies is quickly moved to the cell surface within minutes[45,46]. E-selectin, however, is rapidly synthesized *de-novo* and then translocated to the luminal surface of the venular endothelium. The well-known tethering and rolling of leukocytes onto the endothelial surface (Figure 1.3) is mediated by endogenous ligands of E- and P-selectin (such as PSGL-1, ESL-1)[47,48]. L-selectin is constitutively expressed on leukocytes and is shed from the cell surface on cell activation, which is assumed to occur immediately after rolling begins. L-selectin plays an important role in the so-called “secondary tethering” process[49], i.e. the tethering of leukocytes on leukocytes, which are already associated to the endothelium by the interaction with PSGL-1[50,51]. The “secondary tethering” expands the pool of leukocytes attracted to sites of inflammation. After cell activation by a variety of chemo-attractants and activating factors, L-selectin is cleaved at an extracellular cleavage site by metalloproteases within minutes[52,53]. This process is supposed to facilitate the detachment of leukocytes from endothelial cells prior to the migration through the endothelium[54-59].

In the tethering and rolling phase, the velocity of leukocytes is reduced from the microvascular free stream blood flow of 1-10 mm/s (fast rolling) down to ~5 µm/s (slow rolling). E-selectin was reported to be mainly responsible for the slow rolling of leukocytes (5 µm/s). Hence, E-selectin-dependent ”slow-rolling” drastically increases the transit time through the inflamed tissue and enables the activation of leukocytes by chemo-attractants[60].

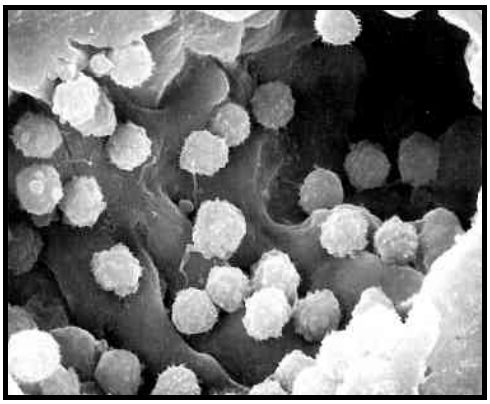


Figure 1.3. Leukocyte adhering to the endothelium[61].

(3) & (4) Integrin activation and firm adhesion

Although rolling is a prerequisite for eventually firm adherence to blood vessels, selectin-dependent adhesion of leukocytes does not contribute to firm adhesion and transmigration unless another set of adhesion molecules is engaged, such as the integrins (Figure 1.4).

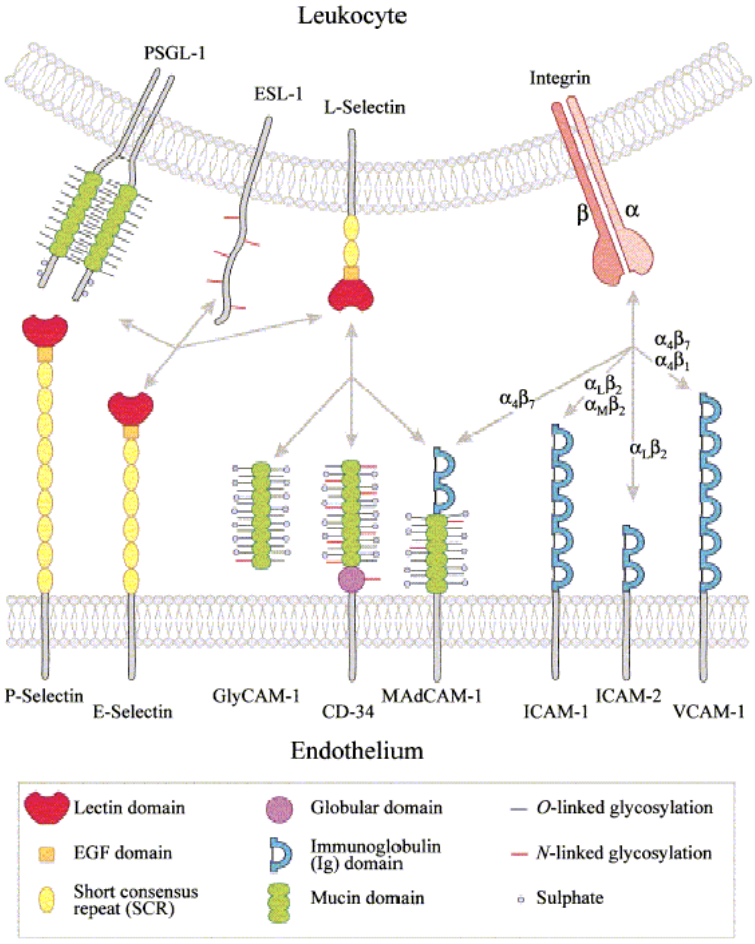


Figure 1.4. Major ligands of selectin and integrin involved in leukocyte recruitment[26].

The activation and deactivation of these integrins at the proper time and place is one of the key events of the adhesion cascade. It has been reported that β_2-integrins (CD18) on the cellular surface of leukocytes are activated and upregulated by exposure to chemoattractants molecules, such as PAF (platelet-activating factor) and IL-8 (interleukin-8)[49,62]. The activated β_2-integrins then recognize their ligands (ICAM-1, VCAM-1) on the endothelial surface and enable the firm adhesion.

CD11/CD18 integrins, specifically CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1), are typical members of the integrin family[63]. They are heterodimers composed of unique α subunits non-covalently bound to a common β subunit (β_2)[64]. The conformational changes of these integrins are activated by chemoattractants. After stimulation with thrombin, histamine or cytokines, the chemoattractants PAF and IL-8 are synthesized by endothelial cells and expressed on the cell surface[65]. The activated integrins then bind to the immunoglobulin superfamily member, such as ICAM-1[66]. ICAM-1 and ICAM-2[65], ligands for the integrins, are constitutively present on endothelia cells *in vitro*[67] and *in vivo*[68]. The constitutive expression can be increased by inflammatory mediators, such as TNF and endotoxin. The interaction of integrin with their ligands enables the firm adhesion of leukocytes onto the endothelium and leads to a flattening which further increases the contact area of leukocytes to endothelium[52,62,69,70].

(5) Transendothelial migration

Transmigration of leukocytes across the endothelial monolayer can be achieved by two processes (Figure 1.5). One occurs by the interaction of integrins located on leukocytes with the platelet-endothelial cell adhesion molecule-1 (PECAM-1) located at the junctions between endothelial cells)[71]. PECAM-1 is a member of the immunoglobulin superfamily. It is expressed at a relatively low level on the surface of leukocytes and platelets but at a higher level (>10^6 molecules per cell) on the endothelium[72]. Anti-PECAM-1 antibody significantly blocks leukocyte transmigration but does not affect the adhesion of neutrophils. The second process in transendothelial migration is the ability of endothelial monolayer to regulate the opening of its junctions, allowing neutrophil transmigration[73].

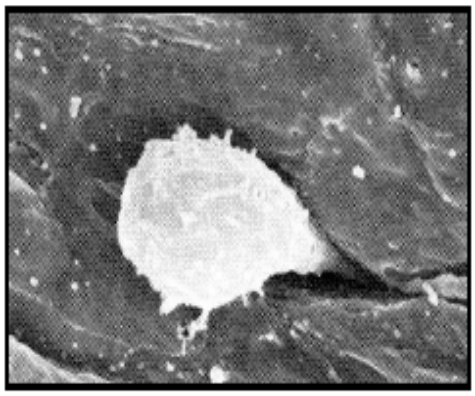


Figure 1.5. Leukocyte transmigrating through the endothelium[61].

1.1.3.3 Biophysics of selectin-based adhesion

The high on-rates (attachment) and high off-rates (detachment) are unique properties of selectins enabling the support of leukocytes rolling under flow[74]. The capture or tethering of leukocytes is closely related to the on-rate, but the actual rolling velocity and the lifetime of selectin-ligand interaction are determined by the off-rate. The relationship between them is that the faster the off-rate, the higher the rolling velocity and the shorter the lifetime of the interaction.

1.1.3.4 The pathophysiological role of selectins

A variety of acute or chronic inflammatory diseases are reported to be caused by the selectin-dependent recruitment of leukocytes into inflamed tissues.

In 1992, “Leukocyte adhesion deficiency type II” (LAD II) was first described in two patients[75]. It is a rare autosomal recessive genetic disorder. These individuals have a point mutation in the gene of a highly conserved GDP-fucose transporter on chromosome 11[76,77], resulting in the insufficient incorporation of fucose into selectin ligands. Consequently, leukocytes cannot bind E-, P-, or L- selectin and patients suffered from infections of the mucosal membranes and skin[78,79]. In some patients, the effect can be overcome by oral administration of fucose[80,81]. The LAD II deficiency clearly demonstrates the importance of interactions between selectins and their ligands in the inflammatory response.

In addition, the tissue and organ damage that result from ischemia, reperfusion[82,83] and asthma[84] can be significantly reduced when the excessive recruitment of neutrophil or eosinophils is averted by a blockade of P- and E-selectin[85]. Some monoclonal antibodies[86-91], carbohydrates[88,92,93], soluble forms of PSGL-1[94] and small molecular antagonists[95,96] showed protective effects in reperfusion injury models.

Recently, soluble E-selectin and ICAM-1 in serum and synovial fluid were recognized as important indicators of the severity of rheumatoid arthritis, allowing early differentiation between mild and severe courses of the disease and providing essential information for therapeutic decisions[97-100]. However, the exact molecular mechanisms of rheumatoid arthritis have not yet been fully elucidated.

Many studies also report that metastasis of some malignant cells follows the pathway of leukocytes[101-118]. The expression of selectins on the endothelium could initiate or even be a prerequisite for the development of metastases. Colon cancers[119-129] and several breast cancer cell lines[130, 131] express selectin ligands, which are correlated with their metastatic behaviour.

E-selectin, compared to P- and L-selectin, plays a particularly important role in this process of metastasis. It mediates the initial rolling of tumor cells on the endothelium, followed by the subsequent firm adhesion involving other, as yet unknown adhesion molecules. An elevated serum level of E-selectin in patients with various types of cancer may reflect the tumor-progression[108,132-137]. P-selectin in the metastasis of tumor cells probably has an identical action mode. Additionally, the stimulation of P-selectin expression on platelets could facilitate the interaction between platelets and tumor cells in the haematogenous metastasis[138-140]. The role of L-selectin in cancer metastasis remains uncertain. It may be involved in the metastasis of malignant lymphoma to distant nodes[141].

In summary, many inflammatory diseases, such as immune complex-dependent acute lung injury[142], sepsis[143,144], skin inflammation[145-148], organ transplantation[149], atherosclerosis, asthma[84], COPD, organ rejection, hemorrhagic shock, thrombosis, rheumatoid arthritis[150], atopic dermatitis, psoriasis[151], diabetes caused microangiopathy, myocardial and cerebral ischemia, and some cancer cell metastasis, are closely related to the selectin-dependent recruitment of leucocytes/tumor cells. Small molecule antagonists or antibodies for blocking the selectin function are promising to be the anti-inflammatory agents. Because inhibition of E-selectin to date appears to have no anti-adhesive and immunosuppressive activity in other organs, inhibition of E-selectin, rather than general selectin inhibition, is an appealing specific therapeutic approach.

1.1.4 E-selectin

E-selectin, also called CD62E or endothelial-leukocyte adhesion molecule- 1 (ELAM-1), was identified as a cytokine-inducible adhesion receptor on endothelial cells. The key role of

E-selectin in the inflammatory cascade is to mediate the initial rolling and adhering of neutrophils, eosinophils, basophils, a subpopulation of T-lymphocytes and monocytes. Human E-selectin is closely related to many acute and chronic inflammatory diseases (see above). Soluble E-selectin is found in human plasma and synovial fluids[152] . Its elevated level in some inflammatory disorders[153] make it the potential marker of disease progress[154].

1.1.4.1 Transient expression of E-selectin by cytokines induction

The transient expression of E-selectin on endothelial cells is induced by cytokines, such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) or liposaccharide (LPS)[155]. Induction by cytokines is conserved across species[156,157]. In contrast, transforming growth factor-ß[158], IL-4[159], corticosteroids[160] and elevation of cAMP[161-163] inhibit the expression of E-selectin. This indicates that the expression of E-selectin is modulated by differernt mechanisms.

E-selectin mRNA and protein are presented within 4-6 hours of induction and decline to the basal levels over the next 12-24 hours, even in the continous presence of the agonist. The rapid decline could be due to the destabilizing sequence of AUUUA in the 3'-untranslated region[21]. Newly-synthesized E-selectin enters the endoplasmic reticulum and Golgi complex to attach the complex N-linked oligosaccharides before reaching the cell suface. For degradation, E-selectin is delivered much more rapidly to lysosomes than most membrane proteins[164-166].

1.1.4.2 Structure features of human E-selectin

Human E-selectin has the common selectin structure and contains six consensus repeat domains (see Table 1.1). The N-terminal C-type lectin domain of E-selectin (118 amino acids in length, CRD)[21,35,167,168] contains four cysteine residues which form two disulfide bridges (Cys19-Cys117 and Cys90-Cys109). The EGF-like domain (36 amino acids in length) contains six cysteine residues that form three disulfide bridges (Cys122-Cys133, Cys127-Cys142 and Cys144 –153) (Figure 1.6). The C-type lectin domain and EGF-like domain were identified as the minimum functional unit of E-selectin[34-36]. The crystal structures of the lectin and EGF-like domains of human E-selectin (*h*E-LecEGF) with or without its natural ligand of sialyl Lewis x (sLe^x) have been reported[30,31] (Table 1.1).

Table 1.1. Brief summary of selectins.

		E-selectin	P-selectin	L-selectin
Structure	Lectin domain	1	1	1
	EGF-like domain	1	1	1
	CR domains	6	9	2
	Transmembrane	1	1	1
	Cytoplamic region	1	1	1
Location		Endothelial cell	Endothelial cell & palatelets	Leukocyte
Recognition		Neutrophils, monocytes, Memory T cells	Myeloid	Cytokine-activated endothelial cells
Crystallization	Apo-LecEGF	Yes	NO	NO
	Glycosylated-LecEGF	Yes	NO	NO
	Deglycosylated-LecEGF	Yes	Yes	Yes
	Deglycosylated-LecEGF+sLe x	Yes	Yes	NO

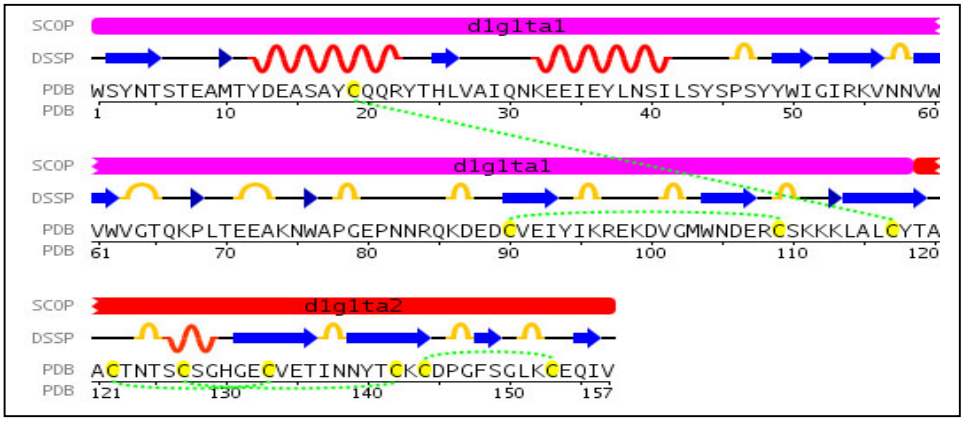


Figure 1.6. The primary protein sequence of E-LecEGF in the crystal structure[30,31].

• Calcium binding sites of E-selectin

Four amino acids of the lectin domain (Glu80, Asn82, Asn105 and Asn106) were identified to be involved in the calcium binding from the crystal structure of *h*E-LecEGF complexed with sLe^x[30,31] and the site-specific mutagenesis of the lectin domain[25].

Human E-selectin may contain more than one calcium-binding site. The equilibrium dialysis method showed that E-selectin contains a high affinity Ca^{2+}-site with a K_D of ~ 3.5 μM. At a

high concentration of Ca^{2+}, additional calcium-binding sites were found[169], coinciding with the crystal data that three calcium ions are presented in the complex of *h*E-LecEGF with sLe^x[30].

The apo-E-selectin was revealed to be sensitive to the partial proteolysis by Glu-C endoproteinase, whereas the holo-E-selectin is not. It suggests that the binding of Ca^{2+} to E-selectin induces a conformational change (in the loop with residues 94-103) and hence facilitates ligand binding.

It was also reported that Sr^{2+} could replace Ca^{2+} and restore the function of all selectin members[170]. Sr^{2+} binds tighter to apo-E-selectin than Ca^{2+}. However, Sr^{2+}-regenerated E-selectin exhibited only 50% ligand binding activity. Ba^{2+} was found to bind to apo-E-selectin five times more strongly than Ca^{2+}, but Ba^{2+}-regenerated E-selectin did not show significant binding activity to HL-60 cells, the carcinoembryonic antigen (CEA) or sLe^x-carrying proteins[169,171].

In summary, Ca^{2+}-binding of E-selectin could induce a minor, but critical conformational change. Perturbations in the conformation of the Ca^{2+}-binding region by either limited proteolysis or substitutions with other metal ions can abolish the function of E-selectin.

- **Structure feature of *h*E-LecEGF complexed with sLe^x**

The molecular interactions between E-selectin and its natural ligand sialyl $Lewis^x$ (sLe^x) were elucidated by the crystal structure of *h*E-LecEGF co-crystallized with sLe^x[30,31]. The interaction was discovered to be an intricate network with mainly electrostatic interaction in nature.

The sLe^x-binding site reveals high conservation between E-LecEGF and P-LecEGF[30,31]. The common feature of this site is the coordination of a calcium ion by the side chains of Glu80, Asn82, Asn105, Asp 106, and the backbone carbonyl of Asp106, and two water molecules to form a pentagonal bipyramid coordination sphere. It is also the structural basis for the metal–dependency of the selectin function. The difference in this binding site of E-LecEGF and P-LecEGF is that the area of Arg97-Glu98-Lys99-Asp100 in E- LecEGF is substituted by Ser97-Pro98-Ser99-Ala100 in P-LecEGF. In E-LecEGF, this region presents a positively-charged surface and Arg97 is stabilized by a hydrogen bond with Asp100. In contrast, this position in P-LecEGF contains uncharged and non-interacting resides. Furthermore, Lys99 in E-LecEGF points away to form the binding sites, whereas Ser99 in P-LecEGF is directed inward.

sLe^x binds to E-LecEGF and P-LecEGF using essentially the same site and with similar conformation of the tetrasaccharide. Compared to the size of the free ligand, the total buried surface area of sLe^x is small (549 Å^2 in E-LecEGF, 501 Å^2 in P-LecEGF). The interactions of sLe^x and E-LecEGF are shown in Figure 1.7 and Figure 1.8. The 3- and 4- hydroxyl groups of Fuc coordinate with calcium and form hydrogen bonds with the residues Asn82, Glu80 and Asn105, which are involved in calcium coordination. The Gal residue of sLe^x forms hydrogen bonds to Tyr94 and Glu92 and the carboxylate group of Neu*N*Ac forms hydrogen bonds to Tyr48.

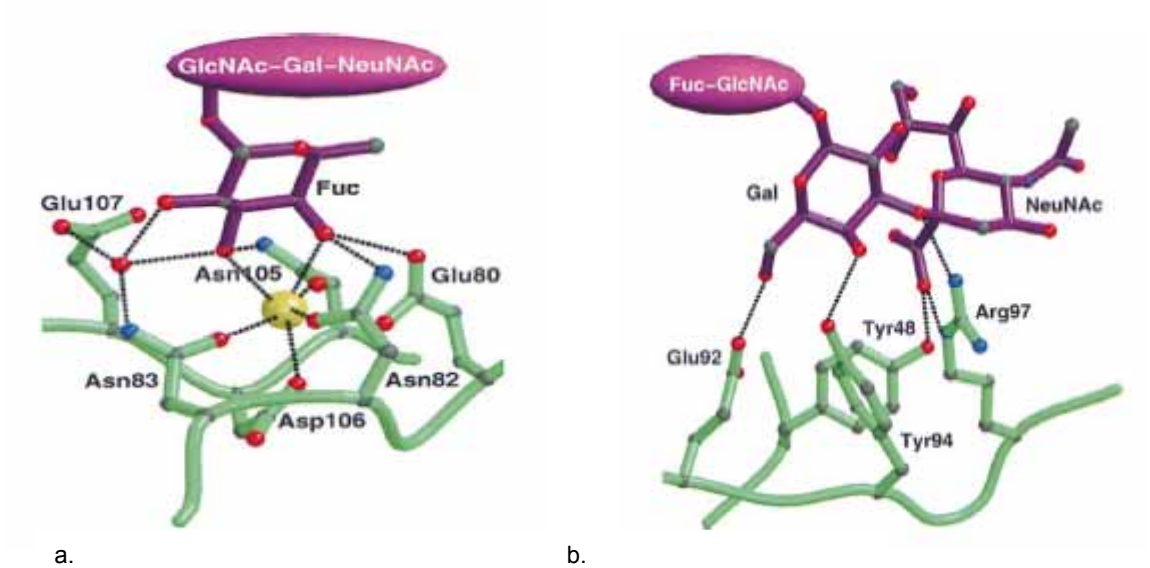


Figure 1.7. Interactions between sLe^x and E-LecEGF in the crystal structure with focus on Fuc (a), Gal and Neu*N*Ac (b) [30, 31].

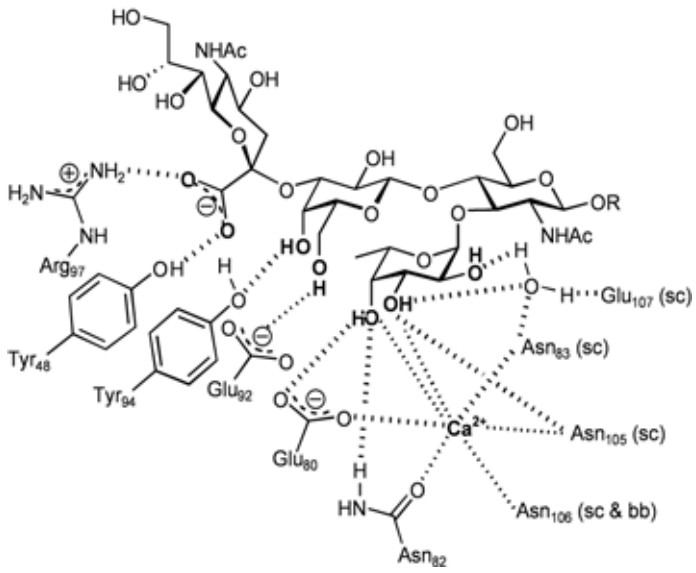


Figure 1.8. The binding mode of sLe^x as determined from the crystal structure[30].

P-selectin binds sLe^x with a K_D of 7.8 mM. E-selectin shows an approximately ten times higher affinity to sLe^x than P-selectin[172]. The structural basis for the higher affinity of E-selectin with sLe^x could be mainly due to the formed salt bridge of Neu*N*Ac in the E-LecEGF/sLe^x complex with the side chain of Arg97 and to the side chain of Tyr48, whereas these salt bridges can not be formed in P-LecEGF.

The folding of the lectin domain in E-selectin is very similar to the lectin domain of the rat mannose binding protein (MBP). The EGF-like domain in E-selectin has the same general fold and arrangement of disulphide bonds as other EGF-like domains. However, only residues 135-139 of the EGF-like domain in E-selectin make contact with the lectin domain by several hydrogen bonds and a limited number of van der Waals contacts.

1.1.4.3 Natural Ligands of E-selectin

The counter-receptors of E-selectin have been identified on neutrophils[173], monocytes[174,175], eosinophils[176], memory T-lymphocytes[146,177,178], natural killer cells[179] and colon carcinoma cells.

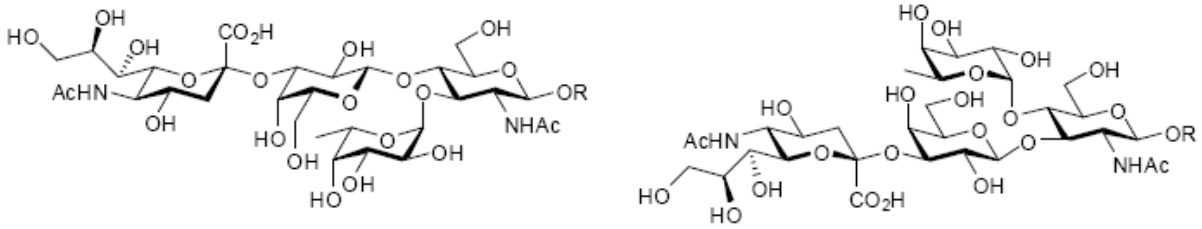
The interaction of E-selectin with its ligands is described as a lectin-carbohydrate interaction. These interactions are characteristically less firm than most described protein-protein interactions, such as antibody-antigen, hormone-receptor interactions.

Many studies indicate that there are clear differences in carbohydrate recognition by the different selectins. E-selectin recognizes the sialylated and fucosylated lactosaminoglycans. A common trisaccharide domain is the minimal binding epitope for E-selectin[180]. The natural ligands of E-selectin include:

(1) Sialyl Lewis^x (sLe^x):
Tetrasaccharide sLe^x (Neu5Ac-α2-3Gal-β1-4(Fuc-α1-3)-Glc*N*Ac) represents a family of α(2,3)sialylation and α(1,3)fucosylation polylactosamines. It is typically found in abundance on circulating neutrophils, monocytes[181-186] and on a small percentage of blood lymphocytes[180,186,187]. It is also highly expressed on a variety of adenocarcinomas[38,188-197]. It is the epitope recognized by E-[198-202], P-[203,204] and L-selectins[205,206], albeit with different affinities. Its structure is shown in Figure 1.9a.

(2) Sialyl Lewis^a (sLe^a):

sLe^a (Neu5Ac-α2-3Gal-β1-3 (Fuc α1-4)-Glc*N*Ac), is a positional isomer of sLe^x[180,206-208]. It binds to E-selectin[106,180] with a higher affinity than sLe^x. sLe^a is not typically expressed by blood leukocytes, but by certain cancer cells, such as the human colorectal carcinoma cell line COLO201. It is a marker of gastrointestinal and pancreatic cancers[209]. The structure of sLe^a is shown in Figure 1.9b.



a. b.

Figure 1.9. The carbohydrate epitope of sLe^x (a) and sLe^a (b).

(3) Sialyl di-Le^x:

Sialyl di-Le^x is a carbohydrate containing sLe^x plus Le^x. It was isolated from HL-60 cells and showed much higher affinity than sLe^x, suggesting that sialyl di-Le^x is responsible for the specificity of E-selectin-dependent adhesion[210]. Its structure is shown in Figure 1.10.

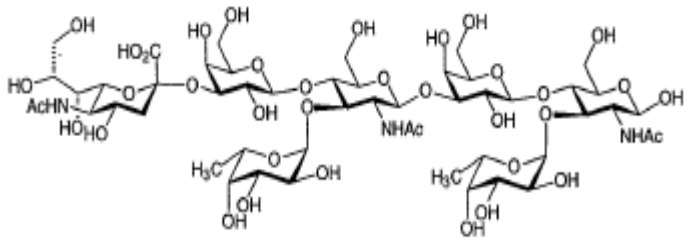


Figure 1.10. The carbohydrate epitopes of sialyl di-Le^x.

(4) PSGL-1

PSGL-1 (P-selectin glycoprotein ligand-1) is a disulfide-linked homodimer of two identical 120 kDa proteins[211,212]. It contains tyrosine sites for sulfation and 15 consecutive Ser/Thr rich decameric repeats for addition of clustered O-linked carbohydrate side chains. α(2,3) sialylation, α(1,3) fucosylation[213,214] are necessary for E-selectin dependent binding[212,214,215]. The sulfation of Tyr on the NH_2-terminus is essential for the recognition by P-selectin[216], but not by E-selectin[217-219]. It is found on human neutrophils, human activated T-cells and HL60 cells. PSGL-1 is a mucin-like protein and shows apparently lower binding affinity to E-selectin than to P-selectin[213].

(5) ESL1 (E-selectin ligand 1)

ESL-1[220-222] is a 150 kDa glycoproteins with sequence similarity to a Golgi-localized polypeptide[223]. It is expressed on HL60 cells, murine neutrophils and myeloid cells lines. Effective function of ESL-1 requires α(1,3) fucosylation[222].

Some other ligands were also identified for the E-selectin, such as the 250 kDa glycoprotein purified from bovine γ/δ T lymphocytes[210], CLA (Cutaneous Lymphocyte antigen) on memory T-cells[224], LAMP-1, LAMP-2, CD44[225,226] and the recently reported Death receptor-3[227] on colon cancer cells.

1.1.5 P-selectin

P-selectin, also known as GMP140 (granule membrane protein)[228], PADGEM (platelet activation-dependent granule to external membrane protein) or CD62P, is a 140 kDa glycoprotein stored in α granules of platelets[228,229], megakaryocytes and Waibel-Palade bodies of endothelial cells. P-selectin mediates the leukocyte rolling in leukocyte recruitment in acute and chronic inflammation[230-232] as described above. Platelet-derived P-selectin could also contribute to hemostatic processes[233] by stimulation of monocytes to express tissue factor[234] or facilitating fibrin deposition during clot formation[235]. Moreover, it could also mediate platelet function in tumor angiogenesis[236,237].

1.1.6 L-selectin

L-selectin, known as gp90^{MEL}, LAM-1[238], CD62L, or TQ1[239], is constitutively expressed on virtually all leukocytes except memory T-cells[240]. It is expressed on the majority of blood borne T- and B-cells, a subset of natural killer cells[240-243], as well as immature hematopoietic cells, including the majority of myeloid colony–forming cells[244,245]. The expression of L-selectin can be downmodulated at the transcriptional level during lymphocyte differentiation from a naive to memory cell phenotype[246] and also rapidly downregulated by a membrane-proximal proteolysis. It recognizes the cytokine-activated endothelial cells in inflammation cascades and is involved in the extravasation of neutrophils, monocytes, possibly eosinophils into inflammatory sites[240,247] and the homing of naive lymphocytes to peripheral lymph nodes[241,248].

1.1.7 Development of selectins inhibitors for therapeutic application

Selectins play an important role in the initial step of cell adhesion in inflammation and in numerous human diseases (see section 1.1.3). Hence, inhibition of selectins is an appealing strategy to develop novel drugs for acute and chronic inflammatory diseases. Four classes of selectin inhibitor have been developed so far and tested in preclinical models and some in clinical trials.

(1) Sialyl Lewis^x-type antagonists

sLe^x inhibits all three selectins at high concentrations and was therefore used to study structure-function relationships and as a sloshing point for the development of drug candidates. Hitherto, sLe^x-type antagonists failed as drug candidates due to their poor pharmacokinetics, low affinity and complex synthesis[249].

(2) Antibodies against selectins

Antibodies against selectins have been developed and humanized for therapeutic use. DREG-55 (anti-L-selectin antibody)[250] is conducting a phase-II, multicenter, double-blind, placebo-controlled trial for psoriasis and multiple trauma with injuries involving two or more organ systems.

(3) Truncated form of a PSGL-1 immunoglobulin fusion protein

Truncated PSGL-1 showed good affinity and pharmacokinetics as selectin inhibitor[251]. Its production in mammalian cells co-transfected with fucosyl transferase and core-2 Glc*N*Ac transferase is complex and expensive. It entered clinical trials which were, however, discontinued.

(4) Small molecule inhibitors of selectins (glycomimetics)

Mimics of sLe^x were developed by substitution of sugar moieties with other moieties without affecting the pharmacophores' presentation. Based on this strategy, several antagonists of E-selectin have been developed[249,252]. TBC-1269, a low molecular weight dimeric glycomimetic, started phase II study in 2002 for childhood psoriasis and for asthma[253,254]. The anti-inflammatory effects of this mimetic are still contradictory[255]. In addition, GMI-1070 (a glycomimetic) commenced phase I clinical trial for vaso-occlusive crisis of sickle cell disease in 2008.

1.2 Recombinant Protein Expression System

Protein expression is an important tool for research and development in the life sciences. Prokaryotic (bacterial) and eukaryotic (usually yeast, insect cells or mammalian cell) systems are two main systems used in the expression of recombinant proteins. The features of these expression systems[256] are shown in Table 1.2. The choice of which system to use must take into account the protein target (including solubility, function and the amount needed) and time involved.

1.2.1 Prokaryotic recombinant protein expression system

Very rapid cell growth, easy expression by induction and simple purification step are the advantages of prokaryotic recombinant protein expression systems. Many commercial kits are available. However, most proteins expressed in prokaryotic systems are insoluble in inclusion bodies and are very difficult to be recovered as functional proteins. Post-translational modifications cannot be accomplished[257,258], which may affect the activity, stability or response to antibodies of the protein.

Escherichia coli (*E. coli*) is one of the most widely used hosts for production of heterologous proteins. Its genetics are better characterized than any other microorganism. Recent progress in the fundamental understanding of transcription, translation and protein folding in *E. coli*, together with serendipitous discoveries and the availability of improved genetic tools are making this bacterium more valuable than ever for expression of complex eukaryotic proteins.

1.2.2 Eukaryotic recombinant protein expression system

Eukaryotic recombinant protein expression systems can secrete the protein of interest into the media and post-translational modifications can be carried out, including methylation, sulfation, phosphorylation, lipid addition and glycosylation. These modifications are crucial for the function of expressed proteins. However, eukaryotic cells grow more slowly than prokaryotic cells and the cost is higher than for the prokaryotic system.

1.2.2.1 Insect cell expression system

The insect cell expression system is a higher eukaryotic system than yeast[259]. It is able to carry out more complex post-translational modifications (Table 1.2), including proper

proteolysis, N- and O-glycosylation, acylation, amidation, carboxymethylation, phosphorylation and prenylation. Insect cell expression systems also have good machinery for folding and expression of soluble proteins of mammalian origin[259].

In insect cell expression system, the most commonly used vector system is baculovirus. The transfer of recombinant DNA can be done by direct transfection or co-transfection. For direct transfection, the target gene is incorporated into the viral genome prior to transfection; for co-transfection, the target gene is simultaneously transfected with linearized viral DNA into insect cells. The genomes of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) and the *Bombyx mori* nuclear polyhedrosis virus (BmPV) are widely used for the transfer. The strong virus polyhedrin promoter increases the expression level of heterologous proteins up to 30% of total cell proteins. The signal sequence, such as the honey-bee prepromelittin secretory sequence[260] or gp 67 signal sequence[261], enable the proteins to be secreted from insect cells or targeted to different subcellular locations. The host is usually a lepidopteran insect cell line, such as *Spodoptera frugiperda* (Sf9 and Sf21) and *Trychlopusia ni (*High five™). High five™ cells are suitable for secretion of the recombinant protein[262].

Insect cell expression systems provide significant advantages, such as high expression levels, ease of scale-up and production of proteins with post-translational modifications. Insect cells do not require CO_2 for growth and can be readily adapted to a high-density suspension culture for large-scale expression[262]. The expressed protein is antigenically, immunogenically and functionally similar to the native mammalian protein. However, particular patterns of post-translational processing and expression must be empirically determined for each construct. The complex glycosylation cannot be accomplished in insect cells. To date, several new systems for more robust and convenient application of baculovirus-based protein expression in insect cells have been invented[263,264], including transient expression systems using a non-viral vector system and novel insect cell lines with “humanized glycosylation function” for better glycosylation of expressed recombinant proteins.

Characteristics	*E. coli*	Yeast	Insect cells	Mammalian cells
Cell Growth	Rapid (30 Min)	Rapid (90 Min)	Slow (18-24 H)	Slow (24 H)
Complexity of Growth Medium	Minimum	Minimum	Complex	Complex
Cost of Growth Medium	Low	Low	High	High
Expression Level	High	Low - High	Low - High	Low - Moderate
Extracellular	Secretion to Periplasm	Secretion to Medium	Secretion to Medium	Secretion to Medium
Expression Protein Folding	Refolding Usually Required	Refolding May Be Required	Proper Folding	Proper Folding
N-linked Glycosylation	None	High Mannose	Simple, No Sialic Acid	Complex
O-linked Glycosylation	No	Yes	Yes	Yes
Phosphorylation	No	Yes	Yes	Yes
Acetylation	No	Yes	Yes	Yes
gamma-Carboxylation	No	No	No	Yes
Yield (mg) (per liter culture)	50-500	10-200	10-200	0.1-100
Success Rate (%) (soluble or functional)	40-60	50-70	50-70	80-95
Project Cost	Low	Low	Middle	High
Advantage	Simple, robust, lowest cost, highest yield	Simple, low cost, good for certain proteins	Relatively higher yield, better PTM	Natural protein configuration, best PTM
Disadvantage	No PTM	Longer time, less PTM	Longer time, higher cost	Highest cost, lower yield
Recommended Use	Antigen protein, Protein standards, Functional proteins	Proteins with glycosylation, Vaccine, Secreted form, Alternative to insect cell system	Proteins with glycosylation, Assay standards, Secreted form, Alternative to yeast system	Functional study, PTM study, Assay standards, Characterization

Table1.2. Comparison of expression systems of *E.coli*, yeast, insect and mammalian cells[256]. PTM: Post-translational modification.

1.2.2.2 Mammalian cell expression system

The mammalian cell expression system has become the dominant system used to produce eukaryotic recombinant proteins and antibodies, since this system has the unique ability to control recombinant protein qualities, including protein folding, disulfide bond formation and post-translational modifications (most are glycosylation)[259]. These are critical features for conformation and activity of the recombinant protein. Compared to other expression systems, yield, time and costs are greater in mammalian systems, although significant progress has been made, especially in preparative scale protein production[265,266]. Transient expression (an episomally replicating plasmid lost gradually) and stable expression (stably integrated copies of the transgene into the host genome) can be carried out for the recombinant protein expression. CHO, BHK and myeloma cells are commonly used for stable protein expression, COS cells for transient expression[267], NS0, HEK-293, NIH3T3 and other cell lines are also used in industry and research.

1.2.3 Post-translational modifications: glycosylation

1.2.3.1 Glycosylation in eukaryotic cells

Glycosylation is the most common form of post-translational modification in eukaryotic cells. Secreted proteins, membrane proteins and proteins targeted to vesicles or certain intracellular organelles are likely to be glycosylated[259]. It is estimated that more than half of all human proteins are glycoproteins. These glycoproteins are involved in a wide range of biological functions, such as receptor binding, cell signalling, immune recognition, inflammation and pathogenicity. The carbohydrate components of glycoproteins affect the functionality of the molecule by determining the protein folding, oligomer assembly and secretion processes. Glycosylation also affects solubility and prevents aggregation of proteins. The oligosaccharides or sugar chains of glycoproteins are covalently linked to proteins. The predominant sugars found on human glycoproteins include galactose (Gal), mannose (Man), fucose (Fuc), *N*-acetylgalactosamine (Gal*N*Ac), *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylneuraminic acid (the human form of sialic acid). Attaching the sugars through the process of glycosylation is the most extensively post-translational modification mode in eukaryotic cells[259].

Mammalian glycoproteins have three major types of oligosaccharides (glycans): N-linked, O-linked and glycosylphosphatidylinositol (GPI) lipid anchors. The most common and best studied is N-linked glycosylation. In this glycosylation pattern, oligosaccharides are uniquely added to asparagine in Asn-X-Ser/Thr recognition sequences of proteins[259]. It occurs

co-translationally in the lumen of the endoplasmic reticulum and continues in the Golgi apparatus. O-linked glycosylation involves either simple oligosaccharide chains or glycosaminoglycan chains to the hydroxyl group of serine or threonine[268]. It occurs post-translationally in the Golgi apparatus.

The attachment of sugars is catalyzed by specific glycosyltransferases. The specific sugars attached to an individual protein depend on the cell type and the physiological status of the cell. However, eukaryotic expression systems do not maintain complete glycosylation under high-volume production conditions[259].

(1) Glycosylation in yeast

The yeast expression system offers a simple production process with high yield, powerful secretory pathways and limited post-translational modifications. The glycosylation patterns are obviously different from mammalian cells. Yeast cells add the non-human type of high-mannose structures to proteins in N-glycosylation pattern (Figure 1.11). Although these structures may not be immunogenic, the expressed glycoprotein has a short half-life. And there is also evidence that different O-glycosylation patterns occur in yeast cells[269].

(2) Glycosylation in insect cells

Insect cells put on shorter mannose structures to the expressed proteins than yeast does. The nature of N-linked glycosylation in insect cells (Sf21, Sf9, High Five™) is dependent on the feature of expressed proteins and the host cell line[259]. N-linked glycans are generally of the high-mannose type, which are not identical to mammalian cells[259] (Figure 1.10).

N-linked glycosylation modified by *Drosophila* is less complex, not trimmed and sialylated. Proteins expressed from *Drosophila* have high mannose contents. High Five™ cells, but not Sf9 cells, modify many glycoproteins with a core alpha (1,3)-fucose[270], which is also found in the glycoproteins of several parasitic worms. Mimic™ Sf9 insect cells, modified Sf9 cells, can stably express a variety of mammalian glycosyltransferases. These enzymes allow for production of the biantennary, terminally sialyated N-glycans from insect cells. Therefore, Mimic™ Sf9 insect cells are used to produce more mammal-like proteins[259].

(3) Glycosylation in mammalian cells

N-linked glycoproteins expressed by mammalian cells contain the standard branched structures composed of mannose, galactose, *N*-acetylglucosamine and neuramic acids

(Figure 1.11). O-linked glycoproteins contain various numbers of sugars, including galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine and neuramic acids.

CHO cells, the system most commonly used today, glycosylate protein close to human cells. However, it does not maintain complete glycosylation under the production conditions[259].

(4) Glycosylation in plant cells

N-glycan structures of human proteins expressed in plants are often simple, without the galactose and terminal sialic acids (Figure 1.11). The presence of alpha 1-3 linked core fucose and xylose residues are potentially immunogenic[271, 272].

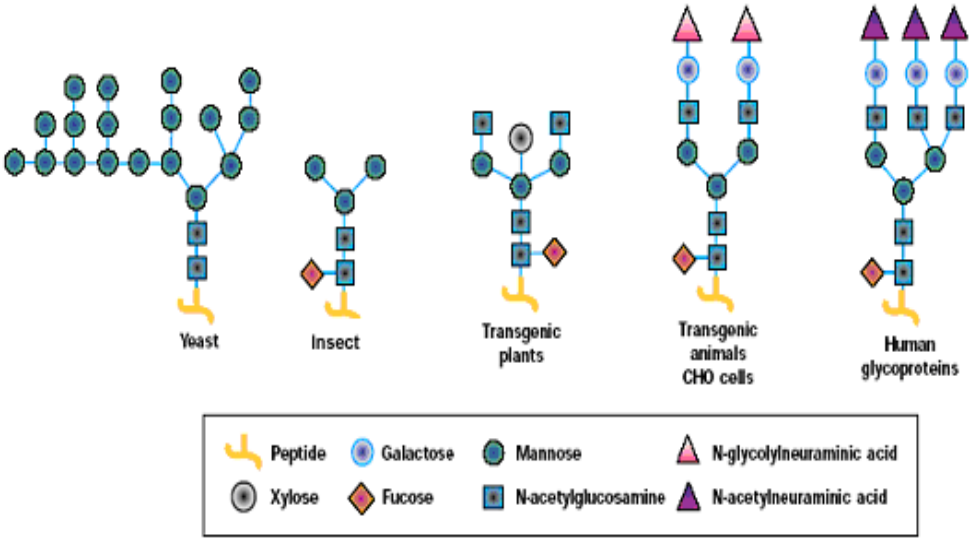


Figure 1.11. Glycosylation in different expression system[259].

1.2.3.2 Enzymes in analysis of N-linked glycosylation

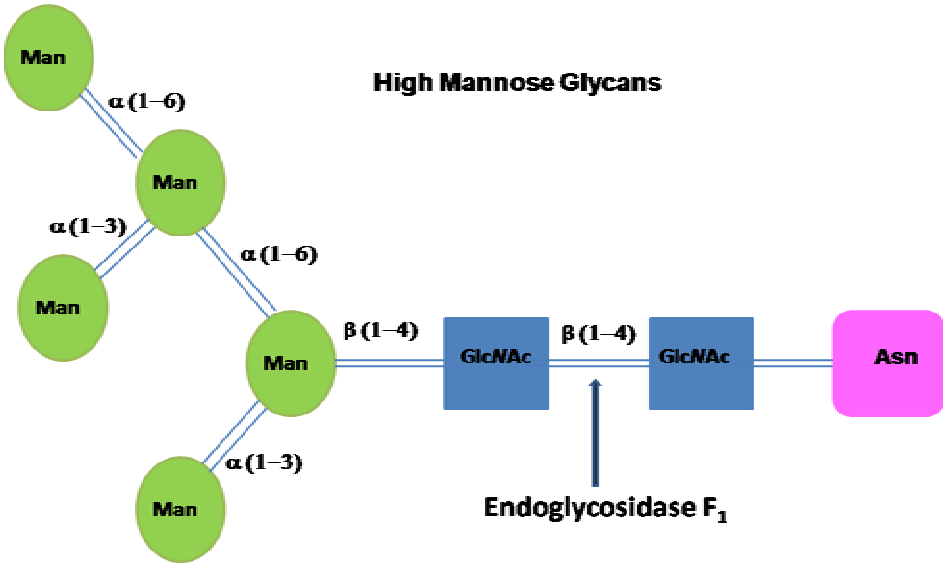
Several protocols for carbohydrate analysis of glycoproteins have been established[273]. Specific deglycosidases have proven useful in the elucidation of the structures and biosynthetic pathways of biologically important glycoproteins. For N-linked glycans, endoglycosidase D, endoglycosidase H, endoglycosidase F, Peptide-N-glycosidase-F (PNGase F) and N-glycosidase A are widely utilized. Endoglycosidases D, H and F can cleave various high-mannose glycans. Endoglycosidase D recognizes the unsubstituted-mannosyl residue linked to the innermost β-mannosyl residue by 1-3 linkage[274,275]. Endoglycosidase H cleaves between the N-acetylglucosamine residues of the chitobiose core of N-linked glycans, leaving one N-acetylglucosamine residue attached to the asparagine[276,277]. Endoglycosidases F1, F2, and F3 are responsible for endohydrolysis of the di-N-acetylchitobiosyl unit in high-mannose-content glycopeptides and glycoproteins (Figure

1.12a). They are intended for deglycosylation of the N-linked oligosaccharides from glycoproteins under native conditions. Some glycoproteins are resistant to the traditional deglycosylation methods using PNGase F due to their conformation[278,279]. Endoglycosidases F1, F2, and F3 are less sensitive to protein conformation than PNGase F. Hence they are more suitable for deglycosylation of native proteins. The linkage specificities of endoglycosidases F1, F2, and F3 suggest a general strategy for deglycosylation of proteins that may remove all classes of N-linked oligosaccharides without denaturing the protein. However, they leave one N-acetylglucosamine residue attached to the asparagine of glycoprotein.

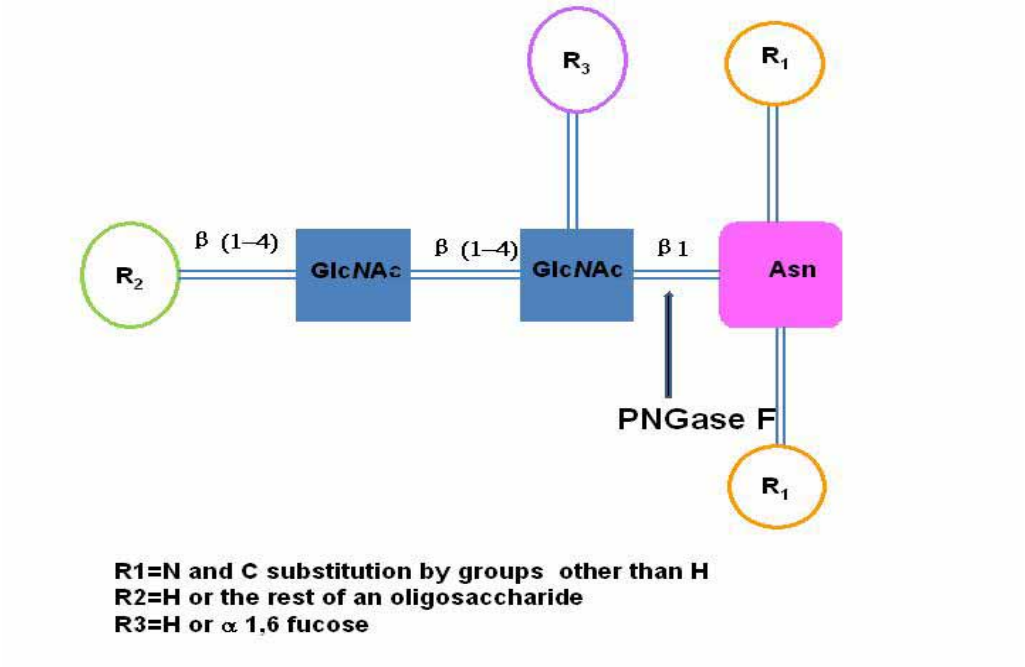
Peptide-N-glycosidase F (PNGase F) is one of the most widely used enzymes for deglycosylation of glycoproteins[280]. The enzyme releases asparagine-linked (N-linked) oligosaccharides from glycoproteins and glycopeptides (Figure 1.12b). The minimum substrate of it is a tripeptide with the glycan-linked asparagine as the central residue. The glycan recognized by PNGase F can be a high-mannose, hybrid or complex type[281]. The reaction mechanism of PNGase F differs from that of endoglycosidases D, H and F. However, N-glycans modified with a fucose linked alpha (1-3) to Asn-Glc*N*Ac are resistant to PNGase F.

N-glycosidase A can cleave all types of asparagine bound N-glycans, including high mannose-, hybrid-, biantennary-, triantennary- and tetraantennary-complex types[282]. It can also cleave a single N-acetylglucosamine residue from the peptide, albeit at a slower reaction rate[283]. In contrast to PNGase F, N-glycosidase A can degrade N-linked glycans carrying a fucose-linked alpha(1-3) to Asn-Glc*N*Ac[284] , which is present in proteins expressed by plant and insect cells. However, in most cases, N-glycosidase A is not able to efficiently remove all susceptible oligosaccharides from glycoproteins[285].

In this thesis, PNGase F and N-Glycosidase A have been used for removing the glycans of *h*E-LecEGF expressed in insect cells and CHO K1 cells.



a.



b.

Figure 1.12. The cleavage sites for endoglycosidase F1 (a) and PNGase F (b).

1.3 Antibody

1.3.1 Structure of the antibody

Antibodies(immunoglobulins) all share a basic structure. It is a capital letter "Y" shaped molecule formed by two heavy chains (H, ~50 kDa/each) and two light chains (L, ~25 kDa/each), linked by disulphide bonds. Each chain contains the variable region (V) and the constant region (C) (Figure 1.13). The variable region (V), composed of 110-130 amino acids, confers the antibody specificity for binding the antigen. It is located in the tips of the "Y" and the amino acids in this region vary greatly between different antibodies. The constant region (C) determines the mechanism used to destroy antigen.

The fragment antigen binding region (Fab fragment), composed of one constant and one variable domain of each of the heavy and the light chains, can be produced with a protease. The fragment crystallizable region (Fc region), composed of two identical protein fragments, is the tail region of an antibody that interacts with cell surface receptors (Fc receptors) and some proteins of the complement system. It allows antibodies to activate the immune system.

Based on the constant region structure and immune function, antibodies are divided into five major classes, IgM, IgG, IgA, IgD, and IgE[286-288]. The most common immunoglobulin class is IgG. Its basic monomer structure is shown in Figure 1.13. Each chain is divided into regions or domains consisting of around 110 amino acid residues. The light chain has two domains and the heavy chain has four. The Fv domain on the N-terminal of both the heavy and light chains is known to be variable in amino acid sequences and called variable domains (V_L and V_H). By contrast, the other domains of antibodies are relatively constant, and are called constant domains (C_L, CH_1, CH_2, CH_3). The variable region is further subdivided into hypervariable (HV) and framework (FR) regions. The HV regions, which directly contact a portion of the antigen's surface, are referred to as complementarity determining region (CDR). Within light and heavy chains, there exist three hypervariable regions (HV 1, 2 and 3) and four FR regions. The FR regions have more stable amino acid sequences than HV regions and separate the HV regions. The FR regions form a beta-sheet structure and serve as a scaffold to hold the HV regions in position.

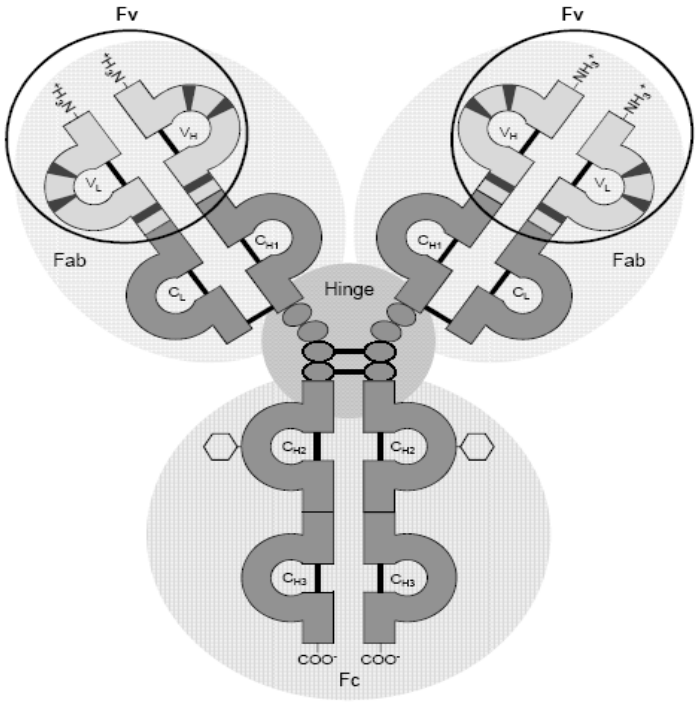


Figure 1.13. Basic antibody structure. Fab: antigen binding region, Fc: crystallizable region, S-S bonds (—) and sugar (hexagon).

1.3.2 Antibody production

1.3.2.1 Monoclonal antibody

Monoclonal antibody (mAb) is an antibody made to target a specific epitope. It is identical and produced from a single parent cell.

The classic method for generation of monoclonal antibodies is hybridoma technology. The technology typically involves fusing myeloma cells with the spleen cells from a mouse immunized with the desired antigen. The production of monoclonal antibodies consists of three stages: (1) immunization to stimulate antigen-specific B-cells *in vivo*, (2) selection of immortalized antigen-specific B-cell clones *in vitro*, (3) propagation of selected B-cell clone *in vitr*o, followed by isolation of antibodies from supernatant.

In contrast to production of polyclonal antibodies, monoclonal antibody production includes a clone selection step *in vitro*, therefore, identical monoclonal antibodies are secreted from one single clone. The produced monoclonal antibody is homogenous, specific to a single epitope and exhibits the defined affinity. The monoclonal antibody-secreting clones produce virtually unlimited quantities of a specific antibody, whereas polyclonal antibodies are limited (200 ml to several liters). Since myeloma cells are professional immunoglobulin-secreting cells and they are able to make post-translational modifications, the produced proteins are properly

glycosylated, which is important for the function of antibodies. However, the generation of monoclonal antibodies is more expensive and time consuming.

1.3.2.2 Polyclonal antibody

Polyclonal antibodies (or antisera) are a mixture of immunoglobulin molecules derived from different B-cell lines. Polyclonal antibodies react against a specific antigen, each of them however recognising a different epitope. Therefore, polyclonal antibodies are a mixture of various antibody types and epitope affinities.

Polyclonal antibodies are obtained from the serum of an immunized animal. They are commonly produced by the following procedures: (1) immunizing animals (goats or rabbits) with an antigen, (2) bleeding the animal periodically (3) extracting the antibodies directly from the serum. The process is relatively inexpensive and large quantities of an antibody can be isolated from a single extraction.

1.3.2.3 Other techniques

Recombinant and phage display technologies have emerged as useful techniques for the generation of antibodies of human origin.

In recombinant technologies, the antibody is produced by placing human genes expressing a particular antibody into a common cell line, such as *E. coli*, in order to reduce immunogenicity, increase affinity or alter the specificity of antibody.

With phage display technologies, large quantities and human original antibodies can be produced but with low affinity.

1.3.3 Applications of antibody

Antibody is a common and essential tool for research applications, including western blotting, immunohistochemistry, immunocytochemistry, enzyme-linked immunosorbent assay (ELISA), immunoprecipitation and flow cytometric analysis. In most immunochemical techniques, polyclonal and monoclonal antibodies are both applicable. Polyclonal antibodies allow the staining of fixed cells, since they bind to the distinct denaturation-sensitive epitopes on one antigen and result in stronger staining. However, due to non-specific or specific binding to irrelevant proteins, “background problems” are common for polyclonal antibodies. Monoclonal antibodies bind only to a single epitope with background seldom low, but they may fail in some techniques due to low avidity, epitope alteration or modification and cross-reactions.

In addition, antibodies are now being designed and used for therapeutic applications, including suppression of the immune system after organ transplantation[289,290], treatment of cancers such as leukemia[291], and inhibition of angiogenesis[292,293]. Monoclonal antibodies achieve their therapeutic effect[294] through the direct or indirect mechanism. For example, they can have direct effects in producing apoptosis or programmed cell death, or blocking growth factor receptors.

1.3.4 Anti-E-selectin antibody

1.3.4.1 Monoclonal antibody 7A9

Anti-E-selectin monoclonal antibody 7A9 (mAb 7A9) produced by hybridoma cell line HB-10135 (ATCC) is an IgG1 subclass antibody. It recognizes the lectin and EGF-like domain of human E-selectin[295]. mAb 7A9 is a functional blocking antibody of E-selectin[296]. The tertiary structure of the Fab fragment of mAb 7A9 has been reported at 2.8Å resolution[297]. The antigen combining site of mAb 7A9 presents a groove resembling the structure of antibodies. The floor and walls of the groove are formed by residues from six complementary determining regions (CDRs) and the framework residues of Fab fragment. The groove is large enough to accommodate the binding site of the lectin domain of E-selectin formed by the loop between β-strands β4 and β5 (Figure 1.6).

In the thesis, mAb 7A9 was produced, purified and coupled to cyanogen activated sepharose 4B matrix for functional purification of the *h*E-LecEGF protein. It was also employed to identify the binding activity of *h*E-LecEGF protein expressed in insect cells and CHO K1 cells.

1.3.4.2 Other anti-E-selectin antibodies

Many monoclonal or polyclonal anti-E-selectin antibodies are available to identify E-selectin, such as BBA1, BBA2, BBA18, ENA1, ENA2 and N18. In the thesis, monoclonal anti-E-selectin N18 antibody (Santa-Cruz biotechnology) was used for identification of the denatured *h*E-LecEGF protein and monoclonal anti-E-selectin/anti-P-selectin antibody BBA1 (R&D system) was utilized for capturing the *h*E-LecEGF protein in a novel capture-binding assay.

1.4 Protein Crystallography

X-ray crystallography is a very powerful tool for determining the three-dimensional structure of macromolecules and macromolecular complexes. It is currently a major contributor to the study of three-dimensional structures of proteins, critical in determining mechanisms of proteins actions. Additionally it can aid the development of novel medications, vaccines and diagnostics. However, the production of soluble, pure protein and good diffraction-quality crystals are clearly the major obstacles in the structural determination of proteins.

1.4.1 Protein sample

The protein sample for crystallization must be homogeneous and pure (≥95% pure by SDS-PAGE). It should have a sufficient amount and exist in an appropriate concentration (1.5 - 200 mg/ml)[298]. SDS-PAGE, gel filtration and dynamic light scattering are suitable methods for assessing the purity and monodispersity of a protein.

1.4.2 Crystallization Method

A primary screening is necessary for finding the crystallization conditions at which protein samples are obtained. The screening is carried out under a rather large number of wide-ranging conditions. These conditions are unrelated to one another and mainly consist of varying ratios of salt, buffer or precipitating agent. The goal of this procedure is to narrow the crystallization conditions. Numerous kits are available, which apply preassembled ingredients in systems and guarantee to produce successful crystallization.

Several different crystallization techniques are used for crystallization of macromolecules[299]. Two of the most commonly used methods, hanging drop and sitting drop methods, fall under the category of vapour diffusion (Figure 1.14). Both methods involve a droplet containing purified protein, buffer and precipitant. The droplet is equilibrated with a larger reservoir containing similar buffers and precipitants in higher concentrations. Initially, the droplet of protein solution contains an insufficient concentration of precipitant for crystallization, but as water vaporizes from the drop and transfers to the reservoir, the precipitant concentration increases to a level optimal for crystallization. Since the system is in equilibrium, these optimum conditions are maintained until the crystallization is achieved[300,301]. The two methods have to be performed in a closed system, that is, the system must be sealed off from the outside using an airtight container or high-vacuum grease between the glass surfaces.

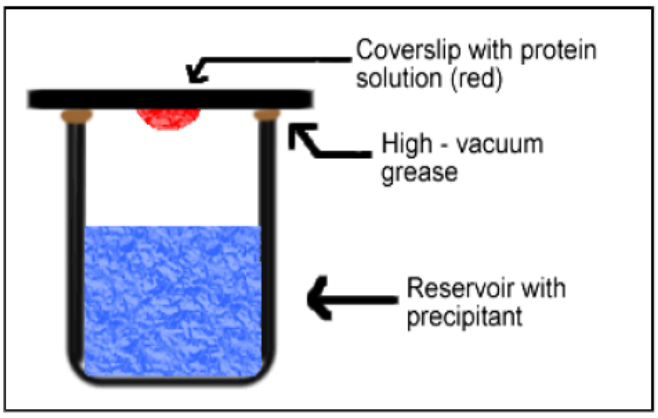
However, even with identical precipitant, protein solution concentrations and volumes for the two set-ups, the experimental results are not always the same.

1.4.2.1 Sitting-drop technique

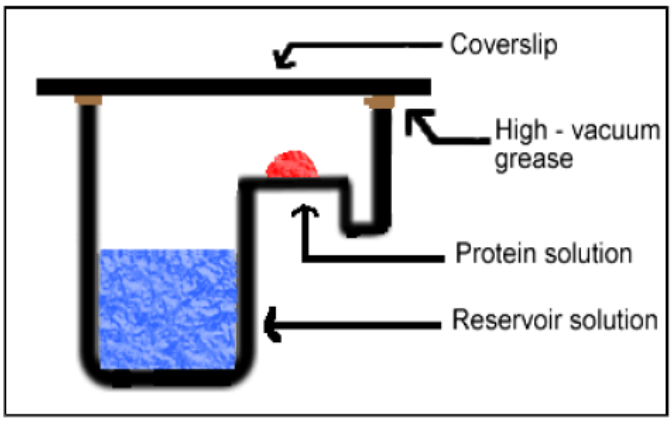
The advantages of the sitting-drop technique are that the automated nano-dispensing system can be used for preparation of the crystallization conditions, and that the minimum samples are consumed. The high-throughput screening is performed by this method. However, protein crystal sediment on the glass or plastic surface in this method can be damaged during the retrieval, and adhered crystals are much more likely to crack during the growth.

1.4.2.2 Hanging-drop technique

Contrary to the sitting-drop method, the protein drop of the hanging-drop method is in the vertical orientation within the system (see Figure 1.14a). The hanging-drop method largely eliminates the problems in the sitting-drop method, since crystal sediment is directed away from the supporting surface toward the liquid–air interface. Freely suspended crystals often have unperturbed facets, show less cracking and are easily retrieved for diffraction studies. Hence, hanging-drops are preferred for the final crystallization trials to obtain the largest, highest quality crystals for data collection. However, obtaining well-formed hanging drops with well-defined surface-to-volume ratios are very difficult. In addition, there is no automated instrument available for high-throughput screening and more protein is consumed in the method, compared with the sitting-drop method.



a.



b.

Figure 1.14. Schematic representation of hanging drop method (a) and sitting drop method (b)[301]. Reservoir solution (blue) usually contains buffer and precipitant. Protein solution (red) contains the same compounds, but in lower concentrations. The protein solution may also contain trace metals or ions necessary for precipitation of particular proteins.

In this thesis, the first crystallization pre-screening of the *h*E-LecEGF protein expressed in CHO K1 cells was performed using the sitting-drop method with an automated nano-dispensing system.

1.5 The Aim

Human selectins (E-, P- and L-selectin) are fundamentally involved in many physiological and pathological processes. They represent an attractive drug target to develop novel drugs against inflammatory diseases. Pan-selectin antagonism is a desired treatment strategy. Inhibition of E-selectin, rather than general selectins, is an appealing specific therapeutic approach, since inhibition of E-selectin appears to have no anti-adhesive and immunosuppressive activity in other, non-targeted organs.

Human E-selectin (*h*E-selectin), a C-type lectin, is transiently expressed on endothelial cells. The key role of *h*E-selectin is to mediate the initial rolling and adhering of leukocytes in cell adhesion. The crystal structure of *h*E-LecEGF complexed with its natural ligand sLe^x, as well as NMR studies of ligand binding, were reported and served for the rational design of *h*E-selectin antagonists. In the past decade, numerous sLe^x analogues or small molecule mimics were designed and synthesized as *h*E-selectin antagonists. However, the binding mode of *h*E-LecEGF complexed with the potent antagonist remains unknown. To develop more potent antagonists, the structure of *h*E-LecEGF co-crystallized with its antagonist is required. Therefore, a sufficient amount of the pure, active and characterized protein is needed for the demands of protein for structure determination.

In addition, synthesized antagonists need to be correctly evaluated in a cell-free assay. To date, the binding affinity of *h*E-selectin antagonists has only been evaluated with *h*E-selectin/IgG or LecEGFFlag. The C-terminal *h*IgG1 tag or Flag tag in *h*E-selectin/IgG or LecEGFFlag protein may interfere with the binding activity of the protein. The binding activity of *h*E-LecEGF has only been screened by a cell-based assay. Thus, a reliable, cell-free assay using the monomeric, soluble and tag-free *h*E-LecEGF is required to evaluate the binding affinity of *h*E-selectin antagonists and the binding activity of *h*E-LecEGF.

In the first part of the thesis, we aim to clone, express and characterize the functional unit of human E-selectin (*h*E-LecEGF) for future structural studies. Insect cell expression systems and mammalian expression systems will be used to achieve this goal.

Furthermore, we aim to functionally purify the *h*E-LecEGF protein using antibody-based affinity chromatography. MAb 7A9, a monoclonal functional blocking anti-E-selectin antibody, will be produced, purified and coupled to the matrix for functional purification.

In the second part of the thesis, we aim to develop a cell-free binding assay using the purified *h*E-LecEGF protein to evaluate the binding affinity of *h*E-selectin antagonists. It will give more accurate and reliable results compared to the previous unstable polymer assay. Several antagonists of *h*E-selectin will be tested in the developed assay.

2. Materials and Methods

2.1 Sequence analysis

2.1.1 DNA sequence

The nucleic acid sequence of human E-selectin was extracted with the entry number of M30640 from GenBank and the amino acid sequence was extracted with the entry number of P16581 from UniProtKB/Swiss-Prot. These sequences were used for primer designs, sequence analysis and prediction of post-translational modification of the lectin and the EGF-like domains of human E-selectin (*h*E-LecEGF).

2.1.2 Prediction of post-translational modification

The post-translational modification of N-glycosylation sites and O-glycosylation sites in *h*E-LecEGF were predicted with the programs of NetNglyc server1.0[302], NetOGlyc3.1 Server[303] and OGPET of Expert Protein Analysis System (ExPASy), respectively.

2.1.2.1 Prediction of N-glycosylation sites

The NetNglyc1.0 server predicts N-Glycosylation sites in human proteins using artificial neural networks that examine the sequence context of Asn-Xaa-Ser/Thr sequons, including Asn-Pro-Ser/Thr. The N-glycosylation sites of *h*E-LecEGF were predicted by submitting the amino acid sequence of *h*E-LecEGF in FASTA format.

2.1.2.2 Prediction of O-glycosylation sites

The NetOGlyc3.1 Server and OGPET program were used for prediction of the O-glycosylation sites of *h*E-LecEGF by submitting the amino acid sequence of *h*E-LecEGF in FASTA format. The NetOGly3.1 produces neural network predictions of mucin type Gal*N*Ac O-glycosylation sites in mammalian proteins. The OGPET algorithm is another algorithm to predict the potential O-glycosylation sites in eukaryotic (non-protozoan) proteins.

2.1.3 Prediction of isoelectric point (pI) and molecular weight (Mw)

The isoelectric point (pI) and Molecular weight (Mw) of the *h*E-LecEGF protein expressed in insect cells and CHO K1 cells were predicted respectively with the program ProtParam[304] of ExPASy by submitting the amino acid sequence of *h*E-LecEGF.

2.2 Cloning, Expression, Purification and Characterization of Human E-LecEGF in Insect Cells

2.2.1 Buffers and Media

• Buffers

TBE buffer 0.5x (45 mM Tris-Borate, 1 mM EDTA)
TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)
TBS buffer (20 mM Tris-HCL, 500 mM NaCl, pH 7.5)
TTBS buffer (20 mM Tris-HCL, 500 mM NaCl, 0.05% Tween 20, pH 7.5)

• Media

LB medium

Bacto tryptone 10.0 g, Bacto yeast 5.0 g and NaCl 10.0 g were dissolved in 950 ml water. After the pH was set to 7.5 with NaOH, volume was adjusted to 1000 ml with water and medium was autoclaved for 20 min at 121˚C. LB medium was stored at 4˚C until use.

LB agar plate

LB agar plates were prepared by adding Bacto agar 15 g per liter LB medium. The medium was autoclaved for 20 min at 121˚C and cooled down to 50˚C. The appropriate antibiotics were then added and 25 ml were poured into each Petri-dish. Solid LB agar plates were enveloped with parafilm and stored at 4˚C until use.

SOC medium

Bacto tryptone 2.0 g, Bacto yeast 0.5 g, 1 ml 1M NaCl, 0.5 ml 1M KCl and 1 ml 2M $MgCl_2$ were dissolved in 95 ml water. After pH was set to 7.0 with NaOH, volume was adjusted to 99 ml with water. Medium was autoclaved for 20 min at 121˚C, cooled down to room temperature (RT) prior to addition of 1 ml sterile filtered 2 M glucose.

Grace's Medium (Invitrogen)

Sf-900 II SFM (Invitrogen)

Express-Five SFM (Invitrogen)

Complete Sf-900 II medium:

Sf-900 II SFM supplemented with 10% FBS (Gibco)

Cryopreservation media:

Sf-900 II SFM supplemented with 10% FBS (Gibco) and 7.5% DMSO (Invitrogen)

RPMI1640-0 medium:

RPMI 1640 medium (Gibco) supplemented with 1 mM Sodium pyruvate (Sigma), 2 mM GlutaMAX (Sigma) and 50U/ml Penicillin/Streptomycin (Sigma).

RPMI1640-2 medium:

RPMI1640-0 medium supplemented with 2% FBS (Gibco, ultra low IgG)

RPMI1640-6 medium:

RPMI1640-0 medium supplemented with 6% FBS (Gibco)

RPMI1640-15 medium:

RPMI1640-0 medium supplemented with 15% FBS (Gibco)

2.2.2 Cloning

2.2.2.1 General DNA methods

(1) OD_{600} measurement

The cell density of *E.coli* cultures was measured by a SmartSpec3000 cuvette spectrophotometer (Bio-Rad) at optical density 600 nm (OD_{600})[305]. Cell numbers were calculated with Equation 1 using a conversion factor at OD_{600} of $5.0X10^8$ cells/ml.

$C = f \times OD_{600}$

Equation 1: Calculation of *E.coli* cell density(c) in cultures by measuring OD_{600} using the conversion factor (f) of *E.coli* ($5x10^8$ cells/ml).

E.coli culture aliquots were diluted to an appropriate concentration in medium. 300 μl diluted cultures or blank (medium) were transferred into plastic semi-micro cuvettes (path length 1cm) and the turbidity was measured at OD_{600}.

(2) Bacterial glycerol stocks for the long time storage

A single clone from the LB agar plate was inoculated into LB medium containing appropriate antibiotics at 37˚C until reach mid-logarithmic phase (OD_{600} of 0.6-0.8). 850 μl cell culture was mixed with 150 μl sterile glycerol in cryotubes and followed by shock-freezing in liquid nitrogen (LN_2) and stored at - 80˚C.

(3) Preparation of $CaCl_2$ competent cells

The $CaCl_2$ competent cells were prepared according to the method from *Sambrook J.*[305]. A single clone of *E.coli* DH 5α was inoculated into 3 ml LB medium and cultured overnight at 37˚C, 300 rpm. 50 ml LB medium was inoculated with 1 ml overnight culture and incubated at 37˚C, 300 rpm until an OD_{600} of 0.5-0.7. Cells were collected by centrifugation at 3000 rpm for 20 min at 4˚C. The cell pellets were then resuspended in 10 ml of 100 mM $CaCl_2$ and incubated on ice for 30 min. After centrifugation at 2000 rpm for 10 min at 4˚C, the cells were resuspended in 1 ml of 100 mM $CaCl_2$ with 20% glycerol and aliquotted at 50 μl in cryotubes. The competent *E.coli* glycerol aliquots were then shock-freezing in liquid nitrogen and stored at - 80˚C.

(4) Agarose-gel electrophoresis

Agarose-gel electrophoresis[305] was performed with appropriate concentration of the gel. The optimal separating resolution for DNA in the range of 0.5-7 kbp is on 1% gel, of 0.4-6 kbp on 1.2% gel, of 0.2-3 kbp on 1.5% gel and of 0.1- 2 kbp on 2% gel.

- **DNA sample preparation**

DNA sample and DNA ladder were diluted to 9 μl with TE buffer (pH 8.0) and mixed with 1 μl 10x Sample loading buffer.

- **Agarose-gel preparation**

0.8 g to 1.6 g agarose was dissolved in 80 ml 0.5x TBE buffer by heating in a microwave to prepare 1-2% agarose-gel. Agarose solution was cooled down to about 50 ˚C prior to adding 0.8 μl ethidium bromide solution (10 mg/ml) and pouring into a horizontal electrophoresis chamber (100x100 mm, 16 pockets).

- **Electrophoresis**

Solid 1-2% agarose-gel containing ethidium bromide (EB, 0.1 μg/ml) was overlaid with 0.5xTBE buffer just prior to use. The gel electrophoresis was run at 80-100 mV (0.8-1 mV/mm) after loading samples into gel pockets. Ethidium bromide stained DNA fragments were visualized under UV light (302 nm) and documented using GelDoc2000 and software Quantity One 4.1 (Bio-Rad).

(5) Quantification of DNA

DNA was quantified by the methods described below.

- **Quantification of the bacterial plasmid DNA by A_{260}/ A_{280} measurement**

The plasmid dsDNA concentration was determined by measuring absorbance at 260 nm (A_{260})[305] in a SmartSpec3000 cuvette spectrophotometer (Bio-Rad). The concentration was calculated with Equation 2 using the conversion factor at A_{260} for dsDNA (50 μg/ml). The purity of DNA was controlled by comparing the absorbance at 260 nm (A_{260}) to 280 nm A_{280}.

$C = f \times A_{260}$

Equation 2: Calculation of the plasmid dsDNA concentration (c) by measuring the absorbance at 260nm (A_{260}) using a conversion factor (f) of dsDNA (50 μg/ml).

The plasmid dsDNA samples were diluted in TE buffer (pH 8.0) to an appropriate concentration. 300 μl samples or blank (TE buffer, pH 8.0) were transferred into a quartz semi-micro cuvette (path length 1cm) and absorbance was measured at 260 nm and 280 nm. Using SmartSpec software, the concentration of plasmid dsDNA and ratios of A_{260}/ A_{280} were calculated.

- **Quantification of the dsDNA by agarose-gel electrophoresis**

Quantification of the bacterial plasmid DNA was also performed on an agarose-gel stained with ethidium bromide (EB)[305]. λ dsDNA standards (0.25, 0.5, 1.0, 2.0, 3.0, 4.5, 6.0, 8.0 and 10.0 μg/ml) and sample in an appropriate concentration in TE buffer (pH 8.0) were run on an agarose-gel containing EB. Using GelDoc2000 and Quantity One 4.1 software, the gel was photographed (UV mode) and the standard calibration curve was drawn to estimate the concentration of the plasmid dsDNA in samples.

2.2.2.2 Construction of the secretion plasmid pFastBacYJS

The secretion plasmid pFastBacYJS was first constructed for secretion of *h*E-LecEGF into the medium in insect cells. The gp67 signal sequence was cloned into the vector pFastBac™1 to get the plasmid pFastBacYJS.

(1) Amplification of the gp67 signal sequence by Polymerase Chain Reaction (PCR)

The forward primer SN1 containing a *Bam*H I restriction site and backward primer SN2 containing an *Xba* I site (Figure 2.1) were designed for amplification of the gp67 signal sequence[261] from the plasmid pAcGP67-A Baculovirus transfer plasmid (Figure 2.2).

Primer SN1: 5'- CGC**GGATCC**ATGCTACTAGTAAATCAGTCA -3'
*Bam*H I

Primer SN2: 5'- CTAG**TCTAGA**CGCAAAGGCAGAATGCGC –3'
Xba I

Figure 2.1. The forward primer SN1 and the backward primer SN2 designed for amplification of the gp67 signal sequence.

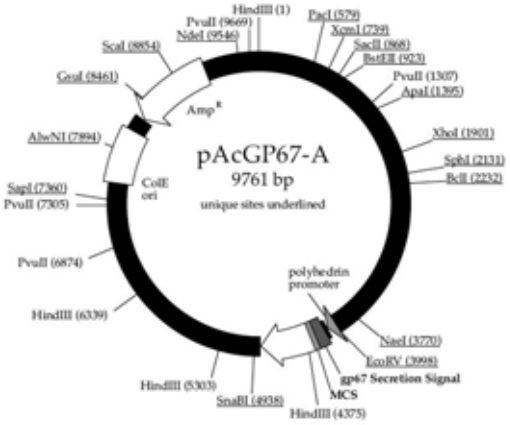


Figure 2.2. Map of the plasmid pAcGP67-A (9761bp).

- **Calculation of the melting temperature and annealing temperature**

The melting temperatures (T_m) of the selected primers were calculated according to the Equation 3[306]. The annealing temperature for SN1 (T_m: 58 ˚C) and SN2 (T_m: 56˚C) primers was determined as 58 ˚C to avoid the non-specific amplification.

T_m (˚C) = 2 ˚C (a + t) + 4˚C (c + g)

Equation 3: T_m determination of a primer containing a: adenine, t: thymidine, c: cytosine and g: guanosine nucleotides.

- **PCR amplification using *Pfu* DNA polymerase**

The PCR method was adapted from the method generated by *S. S. Saiki*[307]. The lyophilized SN1 and SN2 primers (Figure 2.1) were dissolved in sterile water at 100 pmol/µl. For the PCR reaction, primers were then diluted to 10 pmol/µl in sterile water. Mastermix was prepared with the appropriate amounts of template, primers and Pfu DNA polymerase (Promega) in a PCR tube (0.2 ml, GeNunc) on ice according to Table 2.1. The template for the negative control was millipore water.

Table 2.1. Composition of mastermix for 50 µl PCR reaction to get the final concentrations of 200 µM dNTP, 400 nM forward primer/ backward primer, 2.5 U Pfu DNA polymerase in 1x PCR buffer.

	Mastermix
Template DNA (25-250 ng)	5 µl
Forward Primer (10 pM)	2 µl
Backward Primer (10 pM)	2 µl
dNTP Mix (dATP, dGTP, dCTP, dTTP, 10 mM)	1 µl
10x Pfu PCR buffer	5 µl
Pfu DNA polymerase (5 U/µl)	1 µl
Millipore water	34 µl
Total	50 µl

The amplification was performed using the oil-free iCycler thermal cycler (Bio-Rad) with the program shown in Table 2.2. The amplified PCR products were then analyzed by agarose-gel electrophoresis (see section 2.2.2.1) and purified with GenPCR purification Kit (Sigma) according to the manufacturer' s instructions.

Table 2.2. Program of PCR amplification of the gp67 signal sequence.

	Temperature	Time	Cycle
Initial denaturation	94˚C	5 min	1
Denaturation	94˚C	30 sec	30
Annealing	58˚C	1 min	30
Extension	72˚C	1 min	30
Final extension	70˚C	10 min	1
Storage	4˚C	∞	

(2) Restriction enzyme digestion

The purified PCR fragments of the gp67 signal sequence and the plasmid vector pFastBac1 (Figure 2.3) were digested respectively with restriction endonucleases *BamH* I (New England Biolabs, NEB) and *Xba* I (NEB) in the NEB buffer 2, since those two enzymes have 100% activity in the buffer. The reaction mixture was prepared according to Table 2.3 and performed by incubation at 37˚C for 3 h. *BamH* I produces 5'-gatcc- protruding termini and *Xba* I produce 3'-agatc- protruding termini, allowing the specific sticky ligation.

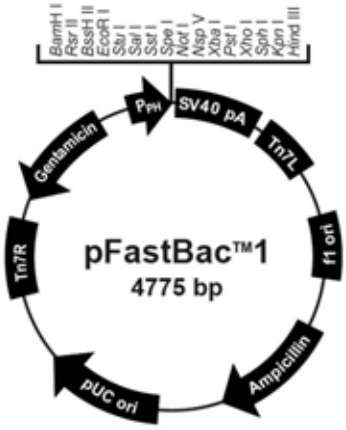


Figure 2.3. Map of the plasmid pFastBac^{TM} 1 (4775bp).

Table 2.3. Composition of *BamH* I and *Xba* I digestions of the gp67 signal sequence fragments and the plasmid pFastBac^{Tm}1.

	gp67 signal fragments	pFastBac^{TM}1
DNA fragments	30 μl	15 μl
10xNEB buffer2	5 μl	5 μl
***BamH* I (10 U/μl)**	1 μl	1 μl
***Xba* I (10 U/μl)**	1 μl	1 μl
BSA (1 mg/ml)	0.5 μl	0.5 μl
Sterilized water	12.5 μl	27.5 μl
Total	50 μl	50 μl

The Digested gp67 signal fragments and the plasmid pFastBac1 were then analyzed by agarose-gel electrophoresis (see section 2.2.2.1) and purified by agarose-gel extraction with the GenEluteTM Gel Purification kit (Sigma) according to the manufacturer's instructions.

(3) Ligation of the gp67 signal sequence into the pFastBac^{TM}1 vector

The ligation mixture containing the gp67 signal fragments and the vector pFastBac^{TM}1 in a ratio of 3:1[308] was prepared according to Table 2.4. The ligation of the digested gp67 signal fragments into the digested pFastBac^{TM}1 was conducted with T4 DNA ligase at 16 ˚C overnight[308].

Table 2.4. Composition for the ligation of the gp67 signal fragment into the pFastBac^{TM}1.

	Volume
10xT4 DNA ligase buffer	2 µl
plasmid pFastBac^{TM}1	2 µl (0.15 µg)
gp67 signal fragments	10 µl (0.45 µg)
T4 DNA ligase (400 U/µl)	1 µl
Sterilized water	5 µl
Total	20µl

(4) Transformation and isolation of plasmids from transformants

- **Transformation**

The plasmid pFastBacYJS was transformed into chemical competent *E.coli* DH5α cells according to the Competent Cell Manual[309]. Two aliquots of 50 µl DH5α competent cells were thawed on ice. 5-10 ng purified plasmids or ligation products and negative control (sterilized water, the same volume to samples) were added and mixed by flicking, respectively. After incubation on ice for 30 min, the cells were heat shocked at 42 ˚C for 45s on a heating block and immediately chilled on ice for 2-3 min. After addition of 500 µl SOC medium, the cells were incubated at 37˚C, 300 rpm for 1h. 200 µl - 400 µl transformed *E.coli* cells were then plated on a LB agar plate containing appropriate antibiotics and incubated (cover-side down) at 37˚C overnight. The transformants were then analyzed to validate the transformation.

- **Isolation of plasmids**

The single colony of transformants was picked from the transformation LB agar plate and inoculated in 3 ml LB medium containing appropriate antibiotics at 37˚C, 300 rpm and overnight. The plasmid DNA from *E.coli* transformant was prepared with GenElute Plasmid Miniprep kit (Sigma) according to the supplier's instruction. The purified plasmid was eluted in 100 µl Millipore water and stored at - 20°C until use.

(5) Analysis of *E.coli* transformants

PCR amplification, restriction endonuclease digestion and DNA sequencing were used to verify the transformants.

- **PCR analysis**

Colony PCR

Colony PCR of *E.coli* transformants was performed according to the pET System Manual[310]. The single colony of transformed *E.coli* was picked from the transformation LB agar plate and suspended in 25 µl 0.1% Triton X-100 by pipetting and vortexing. After incubation at 55˚C for 1h with shaking at 300 rpm, cell lysates were cleared by centrifuge at 12000 rpm for 5min. 10 µl lysate supernatants were taken as the template DNA in PCR for amplifying the target gene following the procedure described in section 2.2.2.2. PCR products were analyzed by agarose-gel electrophoresis (see section 2.2.2.1).

Standard PCR

The purified plasmid from the transformant was diluted in water and served as the template DNA in PCR for amplifying the target gene following the procedure described in section 2.2.2.2.

- **Restriction digestion analysis**

According to Table 2.5, the purified plasmid pFastBacYJS was double-digested with *BamH* I and *Xba* I at 37˚C for 3h, together with the negative control (without restriction enzyme) and the blank control (without plasmid). The digested plasmid was analyzed by agarose-gel electrophoresis (see section 2.2.2.1).

Table 2.5. Set up of double digestions with BamH I and Xba I. Both enzymes are 100% active in NEB buffer 2.

	***BamH* I and *Xba* I**	**Negative control**	**Blank**
10xNEB buffer 2	2 µl	2 µl	2 µl
Plasmid DNA	2 µl	2 µl	-
BSA(1mg/ml)	2 µl	2 µl	2 µl
***BamH* I (10 U/µl)**	1 µl	-	1 µl
***Xba* I (10 U/µl)**	1 µl	-	1 µl
Sterilized water	12 µl	14 µl	14 µl
Total	20 µl	20 µl	20 µl

• **Plasmid DNA sequence verification**

A single clone of transformant was streaked on a LB agar plate containing antibiotics and incubated upside-down at 37˚C overnight. The cultured plate was then sent to Microsynth GmbH sequencing group (Balgach) for DNA sequencing. The plasmid pFastBacYJS was sequenced with the forward primer FastBacf for the forward sequencing. Sequencing results of the gp67 signal fragment were verified by alignment with the gp67 DNA sequence using the Blast programs bl2seq of NCBI server.

Primer FastBacf: 5'- TACTGTTTTCGTAACAGTTTTG -3'

2.2.2.3 Construction of the *h*E-LecEGF expression plasmid pFastBacYJSE

(1) Genomic DNA isolation from CHO-E-sel/IgG cells

For isolation of the genomic DNA, CHO-E-sel/IgG cells expressing human E-selectin fused with a C-terminal human IgG1 tag (human IgG1 Fc fragment) were subcultured in T25 flask and collected by Trypsin-EDTA method. After removal of the culture medium, the cell layer was briefly rinsed with 5 ml of Ca^{2+}/Mg^{2+} free Dulbecco's phosphate-buffered saline (D-PBS, Sigma). The cells were detached by rinse with 2 ml 0.25% (w/v) Trypsin-0.53 mM EDTA solution (sigma) and incubation at 37°C for 1-3 min. 5.0 ml of complete growth medium was then added into the flask and the cells were suspended by gently pipetting up and down. The cells were then collected by centrifugation at 1000 rpm at 4°C for 10 minutes and the genomic DNA was isolated from the harvested cells with WIZARD Genomic Purification Kit (Promega) according to the manufacturer's instructions.

(2) PCR-amplification of the *h*E-LecEGF sequence

The forward primer E-FLE1 containing an *Xba* I restriction enzyme site and a N-terminal flag tag (DYKDDDDK) and the backward primer E-FLE2 containing an *Xho* I site were designed for amplification of *h*E-LecEGF from the genomic DNA of CHO-E-sel/IgG cells (Figure 2.4). The PCR amplification was performed with the annealing temperature of 56 ˚C as described in section 2.2.2.2. The amplified PCR products were analyzed by agarose-gel electrophoresis and purified with GenPCR purification Kit (Sigma) according to the manufacturer's instructions.

Primer E-FLE1:

5′-CTAGTCTAGAGACTACAAG***GACGATGACGACAAG***TGGTCTTACAACACCTCCA-3′

D Y K D D D D K

Xba I **Flag tag**

Primer E-FLE2:

5′- CCGCTCGAGTTACACAATTTGCTCACACTTGA-3′

Xho

Figure 2.4. The forward primer E-FLE1 and backward primer E-FLE2 designed for amplification of the *h*E-LecEGF sequence.

(3) Restriction enzyme digestion

Restriction digestions of the *h*E-LecEGF PCR fragments and the plasmid pFastBacYJS were conducted with *Xba* I and *Xho* I at 37˚C for 3 h with Table 2.6.

Table 2.6. Composition of double digestions of the *h*E-LecEGF PCR fragments and the plasmid pFastBacYJS with *Xba* I and *Xho* I.

	***h*E-LecEGF fragments**	**pFastBacYJS**
DNA fragments	30 µl	15 µl
10xNEB-buffer2	5 µl	5 µl
***Xba* I (10U/µl)**	1 µl	1 µl
***Xho* I (10U/µl)**	1 µl	1 µl
BSA (1mg/ml)	0.5 µl	0.5 µl
Sterilized water	12.5 µl	27.5 µl
Total	50 µl	50 µl

(4) Ligation of the *h*E-LecEGF fragments into the pFastBacYJS

The doubly digested *h*E-LecEGF fragments were inserted into the same digested vector pFastBacYJS by T4 DNA ligase as described in section 2.2.2.2.

(5) Transformation and isolation of the plasmid pFastBacYJSE

The transformation of the plasmid pFastBacYJSE into *E.coli* DH 5α competent cells and isolation of plasmids pFastBacYJSE from transformants were done as described in section 2.2.2.2. A number of transformed clones were then analyzed as below.

(6) Analysis of *E.coli* transformants

- **Colony PCR**

Colony PCR of *E.coli* transformants was performed with E-FLE1 and E-FLE2 primers and Taq DNA polymerase as described in section 2.2.2.2.

• **Restriction digestion analysis**

Double digestions of the plasmids isolated from transformants were performed with *Xba* I and *Xho* I at 37˚C for 3 h as described in section 2.2.2.2.

• **Plasmid DNA sequence verification**

The expression plasmids pFastBacYJSE were sequenced with the forward primer FastBacf for the forward sequencing as described in section 2.2.2.2. The sequencing results of the gp67 signal sequence and the *h*E-LecEGF sequence were verified by alignment using the Blast programs bl2seq of NCBI server.

2.2.2.4 Generation of the recombinant bacmid

(1) Transforming DH10Bac™ *E. coli*

100 µl MAX Efficiency® DH10BacTM competent cells (Invitrogen) was thawed on ice and gently mixed with 5 µl plasmid pFastBacYJSE (1 ng). After incubating on ice for 30 min, the cells were heat-shocked for 45 seconds at 42°C without shaking. The cells were then immediately transferred to ice and chilled for 2 min. 900 µl of S.O.C. Medium was add into the cells and cells were cultivated at 37°C, 300 rpm for 4 h. 200 µl, 300 µl and 500 µl of the cells were plated on transposition LB agar plates containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml Bluo-gal and 40 µg/ml IPTG, respectively. The plates were incubated at 37°C for 48 h. The white colonies were then picked and restreaked on fresh transposition LB agar plates for verifying the phenotype. The restreaked plates were incubated overnight at 37°C. The single colony with a white phenotype on restreaked plates containing Bluo-gal and IPTG was picked for further analysis.

(2) Isolation of the recombinant Bacmid DNA

The single colony confirmed on restreaked plates was picked and inoculated in a 3 ml LB medium containing 50 µg/ml kanamycin, 7 µg/ml gentamicin and 10 µg/ml tetracycline. The culture was grown at 37°C, 300rpm until cells reach stationary phase. The cell pellet of 1.5 ml of bacterial culture was collected by centrifugation at 12, 000 rpm for 1 min and resuspended in 0.3 ml of Solution I (15 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A, filter-sterilize) by gentle vortexing. 0.3 ml of Solution II (0.2 N NaOH, 1% SDS; filter-sterilize) was added, gently mixed and incubated at room temperature (RT) for 5 minutes. The appearance of the suspension changed from turbid to almost translucent. The thick white precipitates of protein and *E. coli* genomic DNA were formed by slowly addition of 0.3 ml of 3 M

potassium acetate (pH 5.5), mixing gently during addition and incubation on ice for 5 to 10 min. After centrifugation at 12,000 rpm for 10 min, the supernatant was gently transferred without any white precipitates to a fresh microcentrifuge tube containing 0.8 ml of isopropanol. After mixing the sample by invertion of the tube a few times and incubation on ice for 5 to 10 min, the sample was centrifuged at 12,000 rpm for 15 minutes at RT. After removing the supernatant, the pellet was washed with 0.5 ml of 70% ethanol by inverting the tube several times. After centrifugation at 12,000 rpm for 5 min at RT, the cell pellet was washed with 70% ethanol once again. The supernatant was then removed as much as possible and the pellet was air dried for 5 to 10 min at RT. The DNA pellet was then dissolved in 40 µl of 1X TE Buffer (pH 8.0) with occasionally gentle tapping of the bottom of the tube. The purified Bacmid DNA was then stored at +4°C for further analysis and transfection into insect cells.

(3) PCR analysis of the recombinant Bacmid DNA

The primer pair of E-FLE1 (Forward) and E-FLE2 (Reverse) (Figure 2.4), and the primer pair of M13 Forward (-40) and M13 Reverse (Figure 2.5) were used to verify the presence of the *h*E-LecEGF sequence in the recombinant bacmid by PCR with Table 2.7 and Table 2.8 as described in section 2.2.2.2. PCR products were then analyzed by agarose-gel electrophoresis.

Primer M13 Forward (-40): 5'- GTTTTCCCAGTCACGAC -3'
Primer M13 Reverse: 5'- CAGGAAACAGCTATGAC -3'

Figure 2.5. The M13 Forward (-40) and M13 Reverse primers for amplification of the transposition sequence in the Bacmid.

Table 2.7. Composition of mastermix for PCR analysis of the recombinant Bacmid DNA.

	Mastermix
Template DNA (100 ng)	1 µl
Forward Primer (10 µM)	1.25 µl
Backward Primer (10 µM)	1.25 µl
dNTP Mix (dATP, dGTP, dCTP, dTTP, 10 mM)	1 µl
10x Taq PCR buffer	5 µl
Taq DNA polymerase (5 U/µl)	0.5 µl
50 mM $MgCl_2$	1.5 µl
Millipore water	38.5 µl
Total	50 µl

Table 2.8. Program of PCR amplification with M13 forward and M13 reverse primers.

	Temperature	Time	Cycle
Initial denaturation	93˚C	3 min	1
Denaturation	94˚C	45 sec	30
Annealing	55˚C	45 sec	30
Extension	72˚C	5 min	30
Final extension	72˚C	7 min	1
Storage	4˚C	∞	

2.2.3 Production of the recombinant baculovirus

2.2.3.1 General cell culture methods

(1) Viability determination and cell counting

The cell number and the viability were determined according to the protocol of Harlow Ed[311]. The mixture of cell suspension and the same volume of trypan blue dye (0.4%) were filled into a Neubauer counting chamber. White living cells and blue dead cells of four big squares (1 mm^2, volume of 0.1 mm^3) were counted with 100X magnification under the microscope (IM, binocular KpI-W10x/18, objective Ph1F10/0.25, Zeiss). The cell density was calculated using Equation 4 and the viability was estimated using Equation 5.

$$c_{cells\ /ml} = n_{viable} \cdot \frac{d \cdot 10^4}{f}$$

Equation 4: Determination of the cell density using a Neubauer counting chamber. c (cells/ml): the cell density of a cell suspension, d: the dilution factor with trypan blue, n_{viable}: the number of viable cells, f : the number of big squares.

$$v\% = \frac{n_{viable}}{n_{total}} \cdot 100\%$$

Equation 5: Determination of the cell viability using a Neubauer counting chamber. v (%): the cell viability of a cell suspension stained with trypan blue, n_{viable}: the number of viable cells, n_{total}: the total number of cells.

(2) Recovering cells from liquid nitrogen storage

Cells were thawed according to a protocol from Stokmaier D[312]. Cells, frozen at –185°C in liquid nitrogen, were quickly thawed at 37°C in a water bath and immediately diluted with 9 ml complete medium in a Falcon tube and pipetted carefully up and down. After centrifugation at 1000 rpm for 10 min, cells were collected and resuspended in supplemented culture medium at a appropriate concentration.

(3) Cell freezing

Cells were frozen by the procedure optimized from Harlow Ed[311]. Briefly, one day before freezing, cells were split 1:2 in fresh medium. Cells in T175 flask were counted, the viability checked (see section 2.2.3.1) and transferred to a sterile, chilled centrifuge tube. After centrifugation at 1000 rpm for 10 min at 4°C, the cells were resuspended in the remaining medium by tapping the tube and were diluted by cold freezing medium (7.5-15%DMSO in appropriate medium) to get ~ $1x10^6$-$1x10^7$ viable cells/ml. 0.5-1.0 ml aliquots were distributed in labeled cryotubes, which were placed in a styropore support. Cells were immediately frozen 1-10 days at – 80°C prior to storage in liquid Nitrogen for infinite time.

2.2.3.2 Cell culture of Sf9 and Hi-5 cells

(1) Recovering cells from liquid nitrogen

Insect cells of Sf9 and High-Five™ (Hi-5) were recovered from liquid nitrogen in the complete Sf-900 II medium (Sf-900 II SFM containing 10% FBS) in T25 flasks (see above). They were then subcultured in Sf-900II SFM medium with no adaptation.

(2) Subculture of adherent cells

When the cell monolayer reaches 80% to 90% confluency, the medium was aspirated and discarded from the monolayer. The appropriate fresh Sf-900II SFM was added to resuspend cells by pipetting the medium across the monolayer with a Pasteur pipette (4-6 ml for T25cm^2 flask and 15 ml for T75cm^2 flasks). The detachment of cells from the surface of flask was observed using an inverted microscope. The viable cells were counted by the trypan blue dye exclusion method (see section 2.2.3.1). Cells were then inoculated at 2 $x10^4$ to 5 x 10^4 viable cells/cm^2 into fresh T flasks and incubated at 27°C ± 0.5°C with loose caps to allow gas exchange. The cells were subcultured approximately 2 to 4 days post-inoculation.

(3) Subculture of suspension cells

Insect cells Sf9 and Hi-5 were adapted to suspension culture from monolayer for the protein production according to the below procedure. Insect cells subcultured in Sf-900 II SFM medium in T flask were dislodged from the bottom of the flask by shaking the flask vigorously two to three times in a wrist-snapping motion. The cell suspension was cultured with approximately $5x10^5$ viable cells/ml at 27.0°C with a stirring rate of 100 rpm. The cells were then subcultured when the viable cell count reaches $1x10^6$ to $2x10^6$ cells/ml (3 to 7 days post-planting). The stirring speed of the cell culture was increased by 5 to 10 rpm with each

subsequent passage until the constant stirring speed reached 120 rpm and cell viability was above 80%. When cells have fully adapted to suspension culture, the cells were subcultured for routine maintenance. Cells were inoculated at $3x10^5$ viable cells/ml in Sf-900II SFM medium and incubated until the cells reach $2x10^6$ to $3x10^6$ viable cells/ml. Suspension cells were subcultured twice weekly. And every 3 weeks, the cell suspension was gently centrifuged at 100 g for 5 min and the cell pellet was resuspended in fresh medium to reduce the accumulation of cell debris and metabolic byproducts.

(4) Cell Freezing

Insect cells Sf9 and Hi-5 were frozen in cryopreservation medium (Sf-900II SFM medium containing 7.5% DMSO and 10% FBS) as described in section 2.2.3.1.

2.2.3.3 Transfection

$9x10^5$ Sf9 cells in 2 ml Sf-900II SFM medium were seeded in a 6-well tissue culture plate per well with the viability > 97% and incubated at 27°C for at least one hour to allow cells to attach. Bacmid DNA:Cellfectin® Reagent was prepared for each transfection sample in 12x75 mm sterile tubes as follows: a) 1-2 µg of purified bacmid DNA was diluted in 100 µl of unsupplemented Grace's Medium (No FBS or antibiotics); b) 6 µl of Cellfectin® Reagent was diluted in 100 µl of unsupplemented Grace's Medium and mixed thoroughly by inverting the tube 5-10 times; c) The diluted bacmid DNA was mixed gently with the diluted Cellfectin® Reagent (total volume is ~210 µl) and incubated for 15 to 45 minutes at room temperature. While DNA:lipid complexes are incubating, the media were removed from the cells and the cells were washed once with 2 ml of unsupplemented Grace's Medium. After removing the washing medium, 0.8 ml of unsupplemented Grace's Medium was added to each tube containing the DNA:lipid complexes and mixed gently. The DNA:lipid complexes were then added to each well and incubated with cells at 27°C for 5 hours. After removing the DNA:lipid complexes, 2 ml of Sf-900 II SFM was added to the cells and the cells were incubated in a 27°C humidified incubator for further 72 hours or until signs of viral infection were observed. The cells were inspected daily for signs of infection beginning at 72 hours after transfection, using an inverted phase microscope at 250-400X magnification. P1 Viral Stock was then isolated as described in section 2.2.3.4.

2.2.3.4 Isolation of P1 viral stock

When the transfected cells showed signs of late and very late stages of infection, the medium (~2 ml) containing virus was collected from 6-well tissue culture plates and transferred to

sterile 15 ml snap-cap tubes. After centrifugation at 500 g for 5 min to remove cells and large debris, the clarified supernatant was transferred to a fresh 15 ml snap-cap tube. This P1 viral stock was stored at 4^{o}C with a final concentration of 2% fetal bovine serum (FBS) and protected from light.

2.2.3.5 Viral plaque assay

In order to determine the titer of a viral stock or isolate a single viral clone, viral plaque assay was performed using the below procedure.

(1) Preparation of Sf-900 plaquing medium

An empty, sterile 100 ml bottle and Sf-900 Medium (1.3X) were warmed in a 40^{o}C water bath. The 4% agarose gel was heated in a microwave oven to be liquefied. The Sf-900 plaquing medium was prepared in a sterile hood by combining 30 ml of Sf-900 Medium (1.3X) and 10 ml of the melted 4% agarose gel in the empty 100 ml bottle. After mixing gently, the plaquing medium was put to the 40^{o}C water bath until use.

(2) Plaque Assay

1x10^{6} Sf9 cells in 2 ml Sf-900II SFM medium were seeded in three 6-well tissue culture plates per well and incubated at room temperature for one hour to allow the cells to settle to the bottom of the plate until the attachment of Sf9 cells inspected by an inverted microscope is at 50% confluence. The 8-log serial dilution (10^{-1} to 10^{-8}) of the clarified viral stock was prepared in Sf-900 II SFM by sequentially diluting 0.5 ml of the viral stock or previous dilution in 4.5 ml of medium in 12 ml disposable tubes. 1 ml of the appropriate virus dilution (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}) was immediately added to cells after removal of the medium from each well in a 6-well tissue culture plate. Two 6-well tissue culture plates were used for the sample plus duplicate for the serial dilution viral stock and one 6-well tissue culture plate was used for the negative control (no virus). After incubation of cells with virus for 1 hour at room temperature, the medium containing the virus was removed from the wells and the wells were replaced with 2 ml of plaquing medium by sequentially starting from the highest dilution (10^{-8}) to the lowest dilution (10^{-3}). The agarose was overlayed to harden for 10-20 min at room temperature before incubating the cells in a 27^{o}C humidied incubator for 7-10 days until plaques are visible and ready to count.

3) Neutral Red staining and titer calculation

Neutral Red staining was performed on day 7-10 post tranfection to facilitate the plaques counting. Neutral Red solution (1 mg/ml) was prepared with sterile and distilled water (cell-culture grade). 0.5 ml of Neutral Red solution was added to each well containing the plaquing overlay and incubated for 1 to 2 hours at room temperature. The plaques were counted after gently removing excess stain with a pipet. Plaques appear as clear spots in a nearly clear gel against a red background. Titer (plaque forming units, pfu/ml) of the viral stock was calculated by the equation 6. The optimal range for counting is 3 to 20 plaques/well in the 6-well plate.

$$\text{Titer (pfu/ml)} = \text{number of plaques} \times \text{dilution factor} \times \frac{1}{\text{ml of inoculum/well}}$$

Equation 6: Estimation of the titer of the recombinant baculovirus.

(4) Plaque purification

Plaque purification was performed to generate a viral stock from a single viral clone. $1x10^6$ Sf9 cells in 2 ml Sf-900II SFM medium were seeded in 6-well tissue culture plates and incubated at room temperature for one hour to attach. The clear plaque in plaque assay was picked using the sterile Pasteur pipette. The agarose plug (containing virus) was transferred to a 1.5 ml microcentrifuge tube containing 500 µl of Sf-900II SFM and mixed well by vortexing. 100 µl of the agarose plug solution was then added to cells in the well of 6-well tissue culture plates and incubated in a 27^{o}C humid incubator for 72 hours. The medium containing the virus from each well (~2 ml) was collected in a sterile 15 ml snap-cap tube and centrifuged at 500g for 5 min to remove the cells and large debris. The clarified supernatant (the plaque-purified viral stock) was stored in a fresh 15 ml snap-cap tube with a final concentration of 2% FBS for further amplification.

2.2.3.6 Preparation of P2 viral stock

2 x 10^6 cells/well Sf9 cells in 2ml (viability > 97%) were plated in a 6-well tissue culture plate and incubated at room temperature for one hour to allow the attachment. The attachment was inspected under an inverted microscope. The appropriate amount of P1 viral stock was added to each well with MOI (Multiplicity Of Infection) of 0.05 and incubated at 27^{o}C for 48-72 hours. MOI is defined as the number of virus particles per cell. The inoculum volume of a viral stock was calculated with a specific MOI using the equation 7. The medium (~2 ml) containing virus from each well was then collected and transferred to sterile 15ml snap-cap tubes. After centrifugation at 500 g for 5 min, the clarified supernatant (P2 viral stock) was collected and

stored with a final concentration of 2% FBS at 4°C or at -80°C for the long-term storage.

$$\text{Inoculum required (ml)} = \frac{\text{MOI (pfu/cell) x number of cells}}{\text{titer of viral stock (pfu/ml)}}$$

Equation 7: Estimation of inoculation volume according to the number of cells, MOI and titer of viral stock.

2.2.3.7 Preparation of P3 viral stock

1 x 10^7 Sf9 cells/well in 10 ml (viability > 97%) was seeded in a T25cm^2 tissue culture flask and incubated at room temperature for one hour to allow the attachment. After verification of the attachment, a appropriate amount of P2 viral stock was added to cells with MOI of 0.05 and incubated at 27°C for 48-72 hours. The viral stock was then collected and stored as described in section 2.2.3.5.

2.2.3.8 Identification of the viral stock by PCR

In order to verify the recombinant virus, the *h*E-LecEGF sequence was amplified by PCR from the viral stock with E-FLE1 and E-FLE2 primers.

2.2.4 Expression of *h*E-LecEGF in insect cells

2.2.4.1 General Protein methods

(1) Polyacrylamide-gel Electrophoresis and staining

- **Polyacrylamide-gel electrophoresis**

The discontinuous polyacrylamide-gel electrophoresis (PAGE) was performed according to the protein methods[313,314]. Proteins were separated in the native PAGE under native condition and in the denaturing SDS-PAGE under reducing or non-reducing conditions.

Reducing SDS-PAGE

Proteins were separated according to their molecular weights on a SDS-PAGE gel by the method of Laemmli[313].

a. Polyacrylamide-gel preparation

The percentage of polyacrylamide in the gel was adjusted according to the molecular weight of the analyzed protein. The best separation resolution for proteins with a molecular weight in the

range of 16-68 kDa was on a 10% gel, of 14-55 kDa on a 12% gel and of 12-43 kDa on a 15% gel.

Inner and outer glass plates, spacers and the comb were rinsed with water and ethanol before assembling. The separating gel solution (375 mM Tris-HCl, x% polyacrylamide, 1% SDS, 0.05% TEMED, 0.03-0.1% APS, pH 8.8) was mixed according to Table 2.9, poured immediately and mounted with water. After polymerization, the stacking gel (125 mM Tris-HCl, 5% polyacrylamide, 1% SDS, 0.1% TEMED, 0.03-0.1% APS, pH 6.8) was prepared, immediately poured and the comb was inserted prior to polymerization.

Table 2.9. Composition of the separating gel x% and the stacking gel.

	Separating gel x%	**Stacking gel**
Acrylamide solution 30% (acrylamide:bisacrylamide 37.5:1)	0.5· x ml	1.7 ml
Water	11.8-(0.5·x) ml	7.2 ml
1.875 M Tris-HCl (pH 8.8)	3.0 ml	
1.25 M Tris-HCl (pH6.8)		1.0 ml
Sodiumdodecylsulfate (SDS) 10% (w/v)	150 µl	100 µl
Ammoniumpersulfate (APS) 10-40% (w/v)	50 µl	33 µl
N,N,N,N'-Tetramethylethylenediamine (TEMED)	7.5 µl	10 µl
	15.0 ml	10.0 ml

b. Protein sample preparation

The low molecular weight marker (Sigma) and protein samples were diluted with 0.25 x sample protein volume 5x sample buffer (200 mM Tris-HCl, 37.5% glycerol, 5% SDS, 250 mM DTT or 2 M β-mercaptoethanol, 0.015% bromophenol blue, pH 6.8) by vortexing. After a brief spin, the samples were boiled for 5 min at 95°C. The denatured samples were then spun down briefly and stored on ice until use.

c. Electrophoresis

The gels were submersed in the running buffer (25 mM Tris, 200 mM glycine, 0.1% SDS, pH 8.6) after complete polymerization and samples were loaded into the gel-pockets using a Hamilton syringe. The electrophoresis was run at a constant current of 30 mA/gel for the stacking gel and of 40 mA/gel for the separating gel in a Mini Protein gel cell (Mini Protean II, Bio-Rad).

Non-reducing SDS-PAGE

The non-reducing SDS-PAGE was done under the same procedure as described for reducing

SDS-PAGE (see section 2.2.4.1), but using the sample buffer composition without reductants of mercaptoethanol or DTT.

Native-PAGE

The native PAGE was prepared under the same procedure as described for reducing SDS-PAGE (see above), but the PAGE gel, gel running buffer and sample buffer contained no SDS and no reductants (mercaptoethanol or DTT). And protein samples were not boiled.

- **Staining methods**

Silver staining

Electrophoretically separated proteins on a PAGE gel slab were silver-stained for the qualitative protein analysis, which has a sensitive detection of protein down to 2-10 ng/band[315].

The PAGE gel was incubated in 125 ml fixation buffer (40% ethanol, 10% acetic acid) in a plastic container for 30 min, while shaking with 70 rpm in the whole procedure and was then soaked in 125 ml sensitizing solution (30% ethanol, 68 mg/ml Sodium Acetate, 2 mg/ml sodium thiosulphate, 0.125% glutaraldehyde) for 30 min. After washing three times with 200 ml water for 5 min, the gel was stained in 125 ml silver solution (2.5 mg/ml silvernitrate, 0.015% formaldehyde) for 20 min and destained twice in ~ 200 ml water for 1 min. The stain of protein bands was then developed in 125 ml developing solution (25 mg/ml sodium carbonate, 0.0075% formaldehyde) for an appropriate time (2-7 min) and stopped in 125 ml stopping solution (14.6 mg/ml $Na_2EDTA{\cdot}2H_2O$) by incubation for 10 min, followed by rinsing once in ~ 200ml water. The stained gel was documented using the GelDoc2000 and Quantity One 4.1 software (Bio-Rad) in the white mode.

Commassie staining

The commassie staining method was performed for the quantitative analysis with the protein detection of 100 ng-1000 ng/band. After electrophoresis, the gel was fixed in the fixing solution (50% methanol, 10% glacial acetic acid) for 30 min to overnight with gentle agitation. Then the gel was stained in staining solution (0.22% Commassie Brilliant Blue G-250, 0.22% Commassie Brilliant Blue R-250, 50% methanol, 10% glacial acetic acid) for 30-40 min with gentle agitation. The destaining of the gel was performed in destaining solution (40% ethanol, 10% glacial acetic acid) in several times washing. The stained gel was then photographed

with the GelDoc2000 and Quantity One 4.1 software (Bio-Rad) in the white mode.

(2) Bradford assay

Bradford assay, originally described by Bradford M.M[316], was performed for quantification of the protein with the standard assay or the micro-assay[317]. The assay is sensitive in the range of 25-200 μg/ml proteins[318].

- **Bradford standard assay**

The BSA standard (0, 20, 40, 60, 80, 100, 120, 160 and 200 μg/ml) and samples of appropriate concentrations in the range of the standard were prepared by dilution with sample buffer (blank). The standards and samples were measured in duplicates or triplicates in the assay. In semi-microcuvettes (path length 1cm), 20 μl BSA standard dilutions, protein samples or blank were mixed with 50 μl of 1M NaOH by pipetting prior to addition of 1ml dye solution (0.01% Commassie Brilliant Blue G250, 10% phosphoric acid 85%, 5% ethanol). After mixing and incubation for at least 5 min, the absorbance was measured within 1h at 595 nm in a SmartSpec3000 cuvette spectrophotometer. Using the SmartSpec or Excel software, the linear regression graph of standards was drawn and the concentrations of the samples were calculated when the correlation coefficient R^2 was above 0.95.

- **Bradford micro-assay**

The BSA standard (0, 100, 200, 400, 600, 800 and 1'000 μg/ml or 0, 20, 40, 60, 80 and 100 μg/ml) and samples were prepared as described for Bradford standard assay (see above). They were measured in duplicates or triplicates. 10 μl BSA standard dilutions, protein samples or blank were added to wells on 96- Microwell V-bottom plates (Nunc), 200 μl dye solution (0.01% Commassie Brilliant Blue G250, 10% phosphoric acid 85%, 5% ethanol) were added then and mixed by tapping the plate gently. After incubation for 10-12 min, the absorbance was measured within 1 h at 595 nm in a Spectramax190 plate reader. Using software Spectramax PRO 4.0, the linear regression graph of the standards was drawn and the concentrations of the samples were calculated when the correlation coefficient R^2 was above 0.95.

(3) Western blotting (Immunoblotting)

A semi-dry transfer method[319] was used for electrophoretically transferring the separated

proteins on a PAGE gel to the nitrocellulose membrane with three different buffers[320].

- **PAGE**

The sample proteins were separated by SDS-PAGE or native PAGE as described above.

- **Electroblotting**

18 sheets of Whatman 3 M absorbent paper and 1 sheet of nitrocellulose membrane (pore size 0.2 m) were cut to the size of the PAGE gel (60mm x 85mm) and the membrane was wet in water first. To assemble the blot sandwich, 6 papers were soaked for a few minutes in Anode buffer I (300 mM Tris, 20% methanol) and piled up on the bottom on anode, followed by 3 papers and the membrane soaked in Anode buffer II (25 mM Tris, 20% methanol), the PAGE gel and 9 papers soaked in Cathode buffer (40 mM 6-aminocaproicacid, 20% methanol) at the top. The electroblotting was performed for 60 min at 15 V using a TransBlot semi-dry transfer cell (BIO-RAD).

- **Staining**

Ponceau S staining of the blotted total proteins

The transfer of separated proteins on the PAGE gel to the membrane was verified by Ponceau S staining. The blotted membrane was stained in 0.1% Ponceau S solution (Sigma) in a plastic container for a few minutes and then destained twice in ~ 100 ml water. The visible Marker bands or the protein bands were marked with a pencil before immunostaining.

Immunostaining of the blotted specific protein

The unspecific binding sites on the blotted nitrocellulose membrane was blocked by incubation with 10 ml blocking buffer (3% BSA in TBS buffer) in a plastic container for 1 h at room temperature or overnight at 4°C, while shaking with 60 rpm in the whole procedure. After washing in 10 ml TTBS-Ca^{2+} buffer (2 mM $CaCl_2$ in TTBS buffer) for 5 min for three times, the membrane was incubated in 10 ml primary antibody solution (appropriate antibodies, 1% BSA, 0.1% NaN_3 in TTBS-Ca^{2+} buffer), overnight at 4°C. The membrane was then washed three times in 10ml TTBS-Ca^{2+} buffer for 5 min and incubated in 10 ml secondary antibody [alkalinephosphatase (AP)-labeled antibodies, 1% BSA, 0.1% NaN_3 in TTBS-Ca^{2+} buffer] for 2 h at room temperature. After washing twice thoroughly in 10 ml TTBS-Ca^{2+} buffer and once in 10 ml TBS-Ca^{2+} buffer for 5 min each washing, the bands of the specific protein recognized by

the primary antibody were developed in 10 ml substrate solution (0.4% NBT/BCIP, 100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 8.8) for an appropriate time. After rinsing the membrane briefly with water, the membrane was dried by the paper tissue and kept in the dark. The immunostained membrane was documented using the GelDoc 2000 and QuantityOne4.1 software in the white mode.

(4) ELISA

The indirect ELISA was performed to determine the presence and quantity of antigen.

- **Coating of antigen**

The appropriate concentration of antigen was coated onto MaxiSorb ELISA plates in 100 μl/well and incubated overnight at 4°C with the cover of the lid and parafilm.

- **Antibody recognition**

After overnight incubation of antigen, plates were washed three times with 200 μl/well washing buffer (2 mM $CaCl_2$, 0.05% Tween 20 in TBS buffer, pH 7.5) and tapped dry on paper towels. The plates were then blocked with 200 μl/well blocking buffer (3% BSA, 2 mM $CaCl_2$, 0.05% Tween 20 in TBS buffer, pH 7.5) for 2 h at RT. Wells were then washed three times with 200 μl/well washing buffer, tapped dry and incubated with 100 μl/well primary antibodies which bind specifically to the antigen in blocking buffer for 2 h at RT. Plates were washed three times with 200 μl/well washing buffer, followed by the incubation for 2 h at RT with 100 μl/well enzyme-linked secondary antibodies which are specific to the primary antibodies.

- **Detection**

After incubation with enzyme-linked secondary antibodies, the plates were washed three times with the washing buffer as previous description. The corresponding substrate was then added to elicit the chromogenic signal. The signal was measured with a spectrophotometer Spectramax 190 plate reader. Table 2.10 showed the composition of color development of the AP-ELISA and HRP-ELISA. The substrate of ABTS substrate solution (Bio-Rad) and stop solution of 2% oxalic acid were used for detection of the horseradish peroxidase (HRP)-labeled secondary antibody and the absorbance was measured at 415 nm. In alkaline phosphatase (AP)-ELISA, 1 mg/ml p-nitrophenyl phosphate disodium was used as the substrate for color development and the chromogenic signal was detected at 405 nm.

Table 2.10. Composition of the detection in HRP-ELISA and AP-ELISA.

	HRP-ELISA	AP-ELISA
Secondary antibody	HRP-labeled anti-mouse antibody, 1:3000 dilute	AP-labeled Goat anti-mouse IgG (Fc specific), 1:3000 dilute
Substrate	ABTS substrate kit (Bio-Rad) Stop buffer: 2% Oxalic acid	1 mg/ml of p-Nitrophenyl phosphate disodium in substrate buffer (10% diethanolamine, 0.5 mM $MgCl_2$, pH 9.8)
Absorption wavelength	415 nm	405nm

2.2.4.2 Optimization of expression

The expression of *h*E-LecEGF was optimized with the below parameters:

a. Cell line:

Two cell lines of Sf9 and High Five™ were used for optimization of the *h*E-LecEGF expression in suspension cultures. Insect cell Sf9 was subcultured in Sf-900II SFM medium, High Five™ was subcultured in Express Five SFM medium.

b. Cell density:

The cells were infected in the mid-logarithmic phase of growth at a density of $1x10^6$, $1.5x10^6$ and $2x10^6$ cells/ml.

c. MOI:

The cells were infected with MOI of 1, 2, 5 and 10, respectively.

d. Time course:

The time course of 24 h, 48 h, 72 h and 96 h post-infection was performed to determine the expression kinetics of the recombinant protein.

2.2.4.3 Detection of the recombinant protein

ELISA and Western-blotting with the monoclonal anti-flag antibody M2 were performed to detect the expression of the recombinant protein *h*E-LecEGF fused with the N-terminal flag tag.

(1) ELISA

ELISA was performed as described in section 2.2.4.1. 150 µl of culture supernatant was coated onto MaxiSorb ELISA plates at 4°C overnight, covered with the lid and parafilm. After

blocking with 3% BSA, the monoclonal anti-flag antibody M2 (SIGMA) was used to recognize the *h*E-LecEGF. Horseradish peroxidase (HRP)-labeled anti-mouse antibody solution (Goat anti-mouse-IgG-HRP) was used as the secondary antibody for detection. ABTS solution (Bio-Rad) was added as the substrate for the color development. 2% oxalic acid was used for stopping the development. The absorbance was read at 415 nm in a Spectramax190 plate reader.

(2) Western-blotting analysis

50 µl of culture supernatant was first separated by SDS-PAGE electrophoresis under reducing conditions. After electro-transferring to the nitrocellulose membrane, the monoclonal anti-flag antibody M2 (SIGMA) with a final concentration of 2 µg/ml was used to recognize the *h*E-LecEGF. The anti-Mouse IgG (whole molecule)−Alkaline Phosphatase antibody (Sigma) was used for detection of *h*E-LecEGF. 0.4% NBT/BCIP was used as substrate for the color development.

2.2.4.4 Production of *h*E-LecEGF in insect cells

The production of *h*E-LecEGF in insect cells was performed according to the optimized expression condition in Sf9 cells and High five^{TM} cells with a appropriate cell density, MOI and post-infection time, respectively. The production was performed at 27°C in several flasks with 100 ml appropriate medium in a 300 ml flask. The supernatant of the culture medium was collected by centrifugation at 500 g for 10 min at 4°C, filtered with 0.22 µM membrane and stored at -20°C for further purification.

2.2.5 Purification and characterization of *h*E-LecEGF

2.2.5.1 Monoclonal anti-E-selectin antibody 7A9 production, purification and coupling to sepharose 4B matrix

(1) Cell culture of hybridoma cell HB-10135

- **Recovering cells from liquid nitrogen**

Hybridoma cell line HB-10135, purchased from American Type Culture Collection (ATCC), expresses the monoclonal functional blocking anti-E-selectin antibody 7A9 (mAb 7A9). The cells were thawed and recovered in fresh RPMI-15 Medium with concentration of 2-4x10^5 cells/ml in a T25 flask and incubated at 37°C (5% CO_2, 100% humidity) using the method described in section 2.2.3.1. The shape of the cells was controlled under the microscope and

the high dense cultures were split.

- **Cell splitting and adaptation to Low FCS Medium**

Cell splitting

After recovering from liquid nitrogen, hybridoma cells were subcultured in a 37°C, 5% CO_2 incubator according to a protocol of Harlow Ed[311]. When cell density reaches $1x10^6$ cells/ml, the cells were split by diluting the cells in the ratio of 1:10 or 1:20 with fresh medium.

Adaptation to low FCS medium

Hybridoma cells were adapted to grow in RPMI-6 medium with 6% FCS and in RPMI-2 medium with 2% FCS from RPMI-15 medium with 15% FCS by initially diluting cell suspension 1:1 with the new medium and continuously diluting cells with increasing concentrations of the new medium. Finally, cells were subcultured in RPMI-2 Medium with 2% FCS.

- **Cell freezing**

Hybridoma cells were frozen in RPMI-15 medium containing 10% DMSO as described in section 2.2.3.1.

(2) Production of the monoclonal antibody 7A9

The production of the mAb 7A9 was following the procedure of Harlow E. et al.[311] in both T-flasks and roller bottles.

- **Production in T-Flasks**

The production of the mAb 7A9 was carried out in RPMI-2 Medium in T175 flasks (50 ml/flask) with inoculation of $5x10^4$ -$5x10^5$ cells/ml of adapted hybridoma cells. The yellowish culture medium containing living cells was collected by centrifugation at 400 g for 5 min. After filtration with Stericup™ (0.22 μM, Millipore), the cleared supernatant was re-inoculated and subcultured with cells to increase the yield of antibodies, or stored at 4°C with addition of 0.02% NaN_3 until the affinity purification. The cell pellet was resuspended in fresh RPMI-2 media and was propagated for the production following the same procedure.

- **Production in Roller bottles**

The production of the mAb 7A9 was also performed in a roller bottle (1700cm^2, Coring) with a rolling speed of 0.5-1 rpm. The adapted hybridoma cells growing in RPMI-2 medium were seeded with a concentration of ~ 5x10^5 cells/ml in 350 ml RPMI-2 Medium in a roller bottle. After a few days culture, the cells were diluted to 450 ml with fresh RPMI-2 medium. The yellowish medium was then collected using the same procedure as described in section 2.2.5.1.

(3) Affinity purification of the monoclonal antibody 7A9

mAb 7A9, an IgG1 antibody, was affinity-purified on the HiTrap Protein G-Sepharose columns (Amersham Pharmacia) by the below procedures.

The Protein G-Sepharose column was run at 4°C by FPLC on a BioLogic workstation with the EZLogic software using degassed buffers at 4°C. After equilibrating the column with 10 ml PBS buffer (pH 7.5), 250 ml clear supernatant (see section 2.2.5.1) diluted with 250 ml PBS buffer was loaded onto one or two HiTrap Protein G HP columns (1ml bed-volume) at a flow rate of 0.5 ml/min. The column was then washed with 10 column-volumes of PBS buffer at a flow rate of 0.5 ml/min, and the bound antibodies were eluted with 10 column-volumes of glycine (100 mM, pH 3.0) at a flow rate of 0.5-1 ml/min. The eluting antibodies were detected by absorbance monitor at 280 nm and 1 ml fractions were collected in 1.5 ml tubes containing 50 µl Tris-HCl (1 M, pH 8.8) for neutralization. The column was neutralized by washing with 5 ml washing buffer and 5 ml water at a flow rate of 1 ml/min and stored in 20% ethanol. The collected fractions were analyzed by SDS-PAGE and pooled for quantification. After addition of 0.02% NaN_3, the antibody solution was stored at 4°C or - 20°C.

(4) Characterization of the monoclonal antibody 7A9

- **Purity analysis**

The purity of the purified mAb 7A9 was checked by 12% SDS-PAGE under reducing condition with silver staining as described in section 2.2.4.1.

- **Verification of the mAb 7A9**

mAb 7A9 is a murine antibody

Western-blotting analysis with Anti-Mouse IgG (Fc specific)–Alkaline Phosphatase antibody

produced in goat (Sigma, A1418) was performed to confirm that mAb 7A9 is a murine antibody.

1 μg mAb 7A9 was run on 12% SDS-PAGE under reducing condition. After transfer to the nitrocellulose membrane, the Anti-Mouse IgG (Fc specific)–Alkaline Phosphatase antibody (Sigma, A1418) with dilution of 1:5000 in TTBS buffer was used to recognize the mouse original antibody. 0.4% NBT/BCIP was used as the substrate for signal detection.

mAb 7A9 is a functional blocking anti-E-selectin antibody

Two western-blotting analysis were performed to verify that mAb 7A9 is a functional blocking anti-E-selectin antibody using the procedure described in section 2.2.4.1. One is carried out with 15 μg of native *h*E-LecEGF as the antigen, which was separated by 8% native PAGE. The other one is performed with 15 μg of reduced and denatured *h*E-LecEGF as the antigen, separated by 12 % SDS-PAGE. In two experiments, 10 μg/ml of mAb 7A9 was used as the primary antibody and the Anti-Mouse IgG (Fc specific)–Alkaline Phosphatase antibody (Sigma, A1418, in 1:5000 dilution) was chosen as the secondary antibody. The color development was done with 0.4% NBT/BCIP.

- **Yield**

The amount of the purified mAb 7A9 was determined by Bradford micro-assay as described in section 2.2.4.1.

(5) Coupling of the monoclonal antibody 7A9 to CNBr-activated Sepharose 4B

In order to functionally purify the *h*E-LecEGF, the purified functional blocking anti-E-selectin mAb 7A9 was coupled to cyanogen bromide activated Sepharose 4B (Sigma, C9142). The below procedure is for the preparation of 2 ml matrix.

- **Preparation of protein**

The purified mAb 7A9 was in the buffer containing glycine and Tris (see section 2.2.5.1). In order to remove these primary amines, which can disturb the coupling, the buffer was changed to the coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.5) by using the Amicon Ultra-4 centrifugal filter devices (Millipore, 10 kDa) at 3500 g for 20 min at 4°C. The amount of mAb 7A9 in coupling buffer was then determined by Bradford micro-assay method described in section 2.2.4.1.

- **Preparation of resin**

0.8 g cyanogen-bromide activated Sepharose 4B matrix was washed and swollen in 1 mM HCl (cold) for at least 30 minutes. A total of 160 ml HCl was added in several aliquots.

- **Coupling**

The cyanogen-bromide activated Sepharose 4B matrix was rinsed with 5 ml coupling buffer (0.1M $NaHCO_3$, 0.5M NaCl, pH8.5) and immediately transferred to the mAb 7A9 solution (14 mg in coupling buffer). The matrix was then incubated with antibody at 4°C overnight on an end-over-end mixer. Bradford micro-assay was used to evaluate the coupling efficiency. After coupling, the matrix was then blocked in blocking buffer (0.2 M glycine, 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.0) for 2 hours at room temperature. After blocking, the matrix was extensively washed with a cycle of high pH (basic coupling buffer) and low pH (0.1 M Na-Acetate, 0.5 M NaCl, pH 4) buffers for five times. The mAb 7A9 coupled resin was then stored at 4°C in storing buffer (20 mM Tris-HCl, 1.0 M NaCl, 0.025% NaN_3, pH 7.5).

- **Characterization**

A small amount of the mAb 7A9 coupled resin was treated with 5x sample buffer (200 mM Tris-HCl, 37.5% glycerol, 5% SDS, 250 mM DTT or 2M -mercaptoethanol, 0.015% bromophenol blue, pH 6.8) by vortexing. After boiling at 95°C for 5 min and briefly spin, the supernatant was analyzed on 12% SDS-PAGE.

2.2.5.2 Purification and characterization of *h*E-LecEGF by anti-E-selectin affinity chromatography

(1) Batch purification with the mAb 7A9 coupled matrix

A batch purification of *h*E-LecEGF expressed in insect cells Hi 5 was performed with 1 ml mAb 7A9 coupled matrix (see section 2.2.5.1) by the following procedure.

The purification was carried out at 4°C in 50 ml centrifuge tubes. The mAb 7A9 coupled Sepharose4B matrix was first equilibrated with 30 ml binding buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.2). The supernatant of *h*E-LecEGF (40 ml, see section 2.2.4.4) was then incubated with 1 ml mAb 7A9 coupled matrix on an end-over-end mixer at 4°C, overnight. After washing the matrix twice in 15 ml binding buffer, the *h*E-LecEGF protein was eluted with 1 ml/aliquot elution buffer (100 mM glycine, pH 2.5) for 5 ml. The elution fractions were immediately neutralized with 60 µl Tris-HCl (1M, pH 8.8). The matrix was then neutralized by

washing twice with 15 ml binding buffer and stored in storing buffer (50 mM Tris-HCl, 1 M NaCl, 2 mM $CaCl_2$, 0.025% NaN_3, pH 7.2) at 4°C for the long-term storage. The collected fractions were stored at 4°C for further characterization.

(2) SDS-PAGE and Western-blotting analysis

The collected fractions from the batch purification with 1 ml mAb 7A9 matrix were analyzed on 15% SDS-PAGE with silver staining as described in section 2.2.4.1. Western-blotting analysis of 1-2 µg reduced elution fractions was performed with the 2 µg/ml of monoclonal anti-flag M2 antibodies (SIGMA) as the primary antibody using the procedure described in section 2.2.4.1.

2.2.5.3 Purification and characterization of *h*E-LecEGF by anti-flag affinity chromatography

(1) Anti-flag affinity chromatography by FPLC

*h*E-LecEGF expressed in Hi-5 and Sf9 insect cells were purified with 2 ml anti-flag M2 monoclonal antibodies agarose (Sigma) according to the manufacture's instructions[268], respectively. The anti-flag M2 monoclonal antibodies agarose was first packed into BioScaleMT-2 column according to the supplier's manual. The purification was done with degassed buffers at 4°C on a BioLogic workstation, using the EZLogic software.

For anti-flag affinity chromatography, the anti-flag column was first equilibrated with binding buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.5) for at least 10 bed-volumes at the flow rate of 1 ml/min until the baseline was reached. The supernatant of *h*E-LecEGF (see section 2.2.4.4) was then loaded onto the column at the flow rate of 1 ml/min. Contaminating proteins were washed out with 5-10 bed-volumes washing buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.5) at a flow rate of 0.5-1 ml/min until the baseline was reached. Then, the protein *h*E-LecEGF fused with the N-terminal Flag tag was eluted isocratically with 3-5 bed-volumes of elution buffer (100 mM Glycine, pH 3.5) at a flow rate of 1 ml/min. Each 1 ml of eluted fractions was collected in a 1.5 ml tube containing 20 µl Tris-HCl (1 M, pH 8.8) for neutralization. The column was then extensively washed with 5-10 bed-volumes washing buffer at a flow rate of 1 ml/min and stored in storing buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, 50% glycerol, pH 7.5) at 4°C for the long-term storage. The collected fractions were analyzed by SDS-PAGE and pooled for further characterization.

(2) Optimization of anti-flag affinity chromatography by FPLC

Since pure *h*E-LecEGF was not obtained by the above anti-flag affinity chromatography (see section 2.2.5.3), the purification strategy was optimized in below parameters.

a. Binding buffer:

PBS buffer (50 mM, 150 mM NaCl, pH 7.5) was used as the binding buffer and washing buffer to instead TBS buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.5).

b. Concentration of salts:

The concentration of 150 mM NaCl in washing buffer was increased to 300 mM and 500 mM respectively to remove the non-specific binding proteins in anti-flag affinity chromatography.

c. Washing volume

10-20 bed-volumes washing buffer was used for the washing step to remove the contaminant proteins in chromatography.

d. Addition of detergents

The addition of 0.05% Tween 20, 0.1% TritonX-100 and 0.5% TritonX-100 respectively in washing buffer were used in chromatography to get the pure protein.

(3) SDS-PAGE and Western-blotting analysis

*h*E-LecEGF purified by the optimized anti-flag affinity chromatography was analyzed on 15% SDS-PAGE with commassie or silver staining as described in section 2.2.4.1. Western-blotting analysis was performed with 2 μg/ml of monoclonal anti-flag M2 antibodies (SIGMA) as described in section 2.2.5.2.

(4) Monomeric identification

The monomeric property of *h*E-LecEGF was analyzed on 15% SDS-PAGE under both reducing and non-reducing conditions with silver staining as described in section 2.2.4.1.

(5) Yield

After the purification with anti-flag chromatography, the amounts of *h*E-LecEGF expressed in Hi-5 and Sf9 cells were determined by Bradford micro assay as described in section 2.2.4.1.

(6) Activity determination

Activities of the purified *h*E-LecEGF, expressed in Hi-5 and Sf9 cells, were identified using both Western-blotting and HRP-ELISA methods.

- **Western blotting**

Western blotting analysis of 1-2 µg of the native *h*E-LecEGF was done using the procedure described in section 2.2.4.1. The *h*E-LecEGF protein was separated on 12% native-PAGE prior to electroblotting, 10 µg/ml of mAb 7A9 (see section 2.2.5.1) was used as the primary antibody to recognize the *h*E-LecEGF protein. The anti-Mouse alkaline phosphatase (AP)-labeled antibody (Sigma) was employed as the secondary antibody. 0.4% NBT/BCIP was used as the substrate for signal detection.

- **ELISA**

3-5 µg of *h*E-LecEGF was analyzed by HRP-ELISA as described in section 2.2.4.1. The primary antibody was 10 µg/ml of mAb 7A9 (see section 2.2.5.1) and the secondary antibody was Horseradish peroxidase (HRP)-labeled anti-mouse antibody solution (GAM-IgG (H+L chain)-HRP, 1:3000 diluted).

(7) Deglycosylation

Deglycosylations of *h*E-LecEGF expressed in Hi-5 and Sf9 insect cells were performed on the analytical scale with Peptide-N-glycosidase F (PNGase F, Roche), Peptide-N-glycosidase A (Roche) and O-glycosydase (Roche), respectively. The deglycosylated *h*E-LecEGF was analyzed on SDS-PAGE under reducing condition.

- **Deglycosylation with PNGase F**

PNGase F, a N-deglycosidase, cleaves all types of asparagines bound N-glycans. And the reaction products are ammonia, aspartic acid in the peptide chain and the complete oligosaccharide[279]. Deglycosylations of *h*E-LecEGF were performed under both native and denaturing conditions at 37°C according to the supplier's manual.

Native condition

The deglycosylations of 3 µg *h*E-LecEGF were performed with 1 U and 2 U of PNGase F in 20 mM sodium phosphate buffer and in Tris-glycine buffer (20 mM Tris, 100 mM glycine) with pH

7.2 and pH 8.6 according to Table 2.11. After incubation for 48h, 72h and 96 h, the deglycosylated *h*E-LecEGF was analyzed on 15% SDS-PAGE under reducing condition.

Table 2.11. Composition of the native deglycosylation condition of *h*E-LecEGF with PNGase F.

	Native condition (37°C)			
20 mM sodium phosphate	pH7.2	pH8.6		
Tris-glycine			pH7.2	pH8.6
Incubation time (48, 72, 96 h)	+	+	+	+
PNGaseF (1U, 2U)	+	+	+	+

Denaturing condition

1 U and 2 U of PNGase F was used to remove the glycans of *h*E-LecEGF (3 µg) in 20 mM sodium phosphate buffer with pH 7.2 and pH 8.6 containing SDS (0.1%, 0.2%), Triton X-100 (1%), NP-40 (1%) or EDTA (25 mM). The reactions were heated at 99°C for 5 min or 10 min prior to the addition of PNGase F (See Table 2.12). After incubation of 72 hours or 96 hours, the deglycosylated *h*E-LecEGF was analyzed on 15% SDS-PAGE under reducing condition.

Table 2.12. Composition of the denaturing deglycosylation condition of *h*E-LecEGF with PNGase F.

	Denaturing condition (37°C)					
20 mM sodium phosphate	pH 7.2	pH 7.2	pH 7.2	pH 8.6	pH 8.6	pH 8.6
Incubation time (72, 96 h)	+	+		+	+	
SDS (0.1%, 0.2%)	+	+		+	+	
NP-40 (1%)	+			+		
Triton X-100 (1%)		+			+	
EDTA (25 mM)	+	+		+	+	
99°C (5 min, 10 min)	+	+	+	+	+	+
PNGas F(1 U, 2 U)	+	+	+	+	+	+

- **Deglycosylation with peptide N-glycosidase A**

N-glycosidase A, a N-deglycosidase, cleaves all types of asparagines bound N-glycans[282], but not able to remove efficiently all susceptible oligosaccarides. It also degrades the N-linked glycans carrying a fucose linked α (1-3) to Asn-Glc*N*Ac[284], which are presented in plant glycoproteins and in some of insect glycoproteins,.

Deglycosylations of *h*E-LecEGF were performed under both native and denaturing conditions based on the manual instructions[321].

Native condition

Deglycosylations of *h*E-LecEGF (3 µg) were performed in 100 mM sodium phosphate buffer with pH 5.0 and pH 6.0 with 1 mU and 2 mU of N-glycosidase A for digestion of 96 hours, respectively (Table 2.13). The deglycosylated *h*E-LecEGF was analyzed on SDS-PAGE under reducing conditions.

Denaturing condition

Under the denaturing condition, *h*E-LecEGF (3 µg) was digested for 96 hours with 1 mU and 2 mU of N-glycosidase A in 100 mM sodium phosphate buffer with pH 5.0 and pH 6.0 containing Triton X-100 (1%) and EDTA (10 mM). The reactions were heated at 99 °C for 5 min or 10 min prior to the addition of N-glycosidase A as described in Table 2.13. The deglycosylated *h*E-LecEGF was analyzed on 15% SDS-PAGE under reducing condition.

Table 2.13. Composition of the native and denaturing deglycosylation conditions of *h*E-LecEGF with N-Glycosidase A.

	Denaturing (37°C)		**Native (37°C)**	
100 mM sodium phosphate	pH 5.0	pH 6.0	pH 5.0	pH 6.0
Incubation time (96 h)	+	+	+	+
Triton X-100 (1%)	+	+		
EDTA (10 mM)	+	+		
99 °C (5 min, 10 min)	+	+		
PNGaseA (1 mU, 2 mU)	+	+	+	+

- **Deglycosylation with N-glycosidase A and PNGase F**

Deglycosylations of *h*E-LecEGF (5 µg) were performed at 37°C with N-glycosidase A and PNGase F under native condition. *h*E-LecEGF expressed in Hi-5 and Sf9 cells was first digested with N-glycosidase A (2 mU) for 96 hours. After dialysis in Dialysis Mini Tubes (MWCO10000, Pierce), *h*E-LecEGF was changed to the digestion buffer of PNGase F. PNGase F (2 U) was then added and incubated for additional 96 hours (see Table 2.14). Digestions of *h*E-LecEGF with PNGase F first and followed with N-glycosidase A were also performed using the same procedure described above. The deglycosylated *h*E-LecEGF was analyzed on 15% SDS-PAGE under reducing condition.

Table 2.14. Composition of the native deglycosylation condition of *h*E-LecEGF with N-glycosidase A and PNGase F.

Native condition (37°C)	
N-glycosidase A (2 mU)	**PNGase F (2 U)**
100 mM sodium phosphate Incubation time: 96 h pH 5.0	20 mM sodium phosphate Incubation time: 96 h pH 7.2, pH 8.6

- **Deglycosylation with O-glycosidase**

The deglycosylation of *h*E-LecEGF expressed in Hi-5 and Sf9 cells (10 µg) was done with 2.5 mU of O-glycosidase (Roche) and 2 mU of Neuraminidase in 20 mM sodium phosphate buffer (pH 7.2) under native condition according to the supplier's instructions. After 72 hours incubation, the deglycosylated *h*E-LecEGF was analyzed on 15% SDS-PAGE under reducing condition.

(8) Glycans detection

Glycans detection of the *h*E-LecEGF and the deglycosylated *h*E-LecEGF was performed with a GelCode Glycoprotein Staining Kit (Pierce) according to the instruction manual[266].

The GelCode® Glycoprotein Staining Kit detects glycoprotein sugar moieties in polyacrylamide gels and on nitrocellulose membranes. The glycols in glycoproteins are oxidized to aldehydes when treated with Oxidizing Reagent (periodic acid). After completing the procedure, the glycols are stained, yielding magenta bands with a light pink or colorless background.

*h*E-LecEGF and deglycosylated *h*E-LecEGF were separated on 15% SDS-PAGE under reducing condition. The gel was then fixed in 100 ml of 50% methanol for 30 min. After washing the gel twice in 100 ml of 3% acetic acid for 10 min by gently agitating, the gel was transferred to 25 ml of Oxidizing Solution and incubated for 15 min. After washing the gel three times with 100 ml of 3% acetic acid for 5 min, the gel was transferred to 25 ml of GelCode® Glycoprotein Staining Reagent for 15 min. Afterwards, the gel was transferred to 25 ml of Reducing Solution for 5 minutes. After washing the gel extensively with 3% acetic acid and then with ultra pure water, glycoproteins appear as the magenta bands. The gel was stored in 3% acetic acid.

2.3 Cloning, Expression, Purification and Characterization of human E-LecEGF in CHO-K1 cells

2.3.1 Buffers and Media

Buffers

HAB buffer: 20 mM Hepes, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.4

HAB20 buffer: 20 mM Hepes, 150 mM NaCl, 20 mM $CaCl_2$, pH 7.4

Media

Ham's F12 complete medium:

Ham's F12 medium (500 ml, Gibco) containing 10% FBS (Invitrogen) and 50 U/ml of Penicillin/streptomycin (Sigma).

Ham's F12 selective medium:

Ham's F12 medium (500 ml, Gibco) containing 10% FBS (Invitrogen), 50 U/ml of Penicillin/streptomycin (Sigma) and 500 μg/ml geneticin (Invitrogen).

DMEM : F12 selective medium:

DMEM:F12 medium (500 ml, Gibco) containing 5% FBS, 50 U/ml of Penicillin/streptomycin (Sigma) and 500 μg/ml geneticin.

2.3.2 Construction of the expression plasmid pYJE

The expression plasmid pYJE was constructed for secretion of the *h*E-LecEGF protein without a tag in CHO K1 cells.

2.3.2.1 Isolation of the plasmid pcDNA3.1(+)

The plasmid pcDNA3.1(+) was kindly offered by Ms.Courtet (Biocenter, University of Basel). The plasmid pcDNA3.1(+) contains a CMV promoter to regulate the expression of recombinant proteins in mammalian cells. Neomycin resistant gene in the plasmid was used to select the transfectants with geneticin in mammalian cells, Ampicillin resistant gene in the plasmid was used to select the transformants with ampicillin in *E.coli* and T7 priming site of it was used for verification of the inserted gene by DNA sequencing (Figure 2.6).

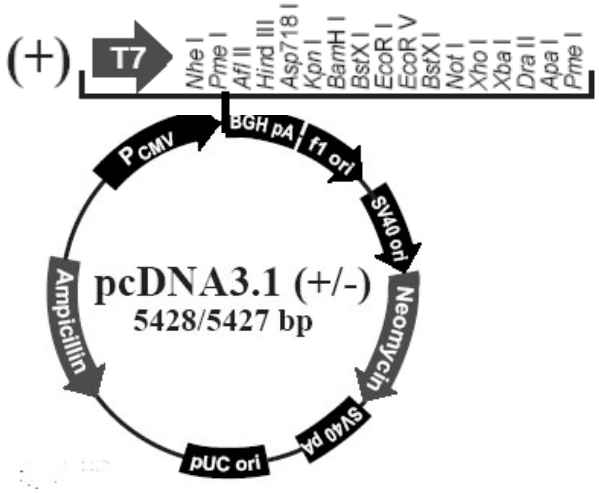


Figure 2.6. Map of the plasmid pcDNA3.1(+).

2.3.2.2 Amplification of the SLE domains of human E-selectin by PCR

The forward primer E1 containing a *Bam*H I restriction endonuclease site and the backward primer E2 containing an *EcoR* I restriction site were designed (Figure 2.6) for the amplification of signal, lectin and EGF-like domains of human E-selectin (*h*E-SLE) from the genomic DNA isolated from CHO cells expressing human E-selecting fused with a C-terminal human IgG1 tag (see section 2.2.2.3).

Primer E1: 5′- CG**GGATCC**ACCATGATTGCTTCACAGTTTCTC-3′
*Bam*H I
Primer E2: 5′- CG**GAATTC**TTACACAATTTGCTCACACTTGA - 3′
*Eco*R I

Figure 2.7. The forward primer E1 and backward primer E2 designed for amplification of the SLE sequence of human E-selectin.

Using the same PCR procedure described in section 2.2.2.3, The SLE sequence of human E-selectin was amplified with the annealing temperature at 56 ˚C. The PCR products were then analyzed by agarose-gel electrophoresis and purified with GenPCR purification Kit (Sigma).

2.3.2.3 Restriction enzyme digestion

Restriction endonucleases *BamH* I and *EcoR* I were used to double digest both PCR product of the SLE fragment of human E-selectin and the vector pcDNA3.1(+). The reactions were performed at 37˚C for 3 h according to Table 2.15.

Table 2.15. Composition of double digestion of the *h*E-SLE fragment and the plasmid pcDNA3.1(+) with *BamH* I and *EcoR* I in NEBuffer-EcoRI. Both enzymes are 100% active in this buffer.

	E-SLecEGF fragment	**PcDNA3.1(+)**
DNA fragment	30 µl	15 µl
10xNEBuffer-EcoRI	5 µl	5 µl
***BamH* I (20 U/µl)**	1 µl	1 µl
***EcoR* I (20 U/µl)**	1 µl	1 µl
BSA (1 mg/ml)	0.5 µl	0.5 µl
Sterilized water	12.5 µl	27.5 µl
Total	50 µl	50 µl

The digested PCR products of the *h*E-SLE fragment and the plasmid pcDNA3.1(+) were analyzed by agarose-gel electrophoresis and purified by agarose-gel extraction with the GenElute^{TM} Gel Purification kit (Sigma) according to the manufacturer's instructions.

2.3.2.4 Ligation of the *h*E-SLE fragment into the pcDNA3.1 (+)

The ligation of the double-digested *h*E-SLE fragment into the vector of pcDNA3.1(+) with the same digestion was done using T4 DNA ligase as described in section 2.2.2.2.

2.3.2.5 Transformation and isolation of the plasmid pYJE

The plasmid pYJE was amplified by transformation of *E.coli* cloning strain DH5α and isolated with GenElute Plasmid Miniprep kit (Sigma) as described in section 2.2.2.2.

2.3.2.6 Analysis of *E.coli* transformants

(1) Colony PCR

Colony PCR of *E.coli* transformants was performed as described in section 2.2.2.2, using E1

and E2 primers (Figure 2.7) and *Taq* DNA polymerase.

(2) Plasmid DNA sequence verification

The plasmid pYJE was sequenced with the primer T7 (5'- TAATACGACTCACTATAGG -3') for the forward sequencing in the Biocenter sequencing group with 4 capillaries machine: ABI3100 Avant. The samples for sequencing were prepared by the below procedure.

- **PCR amplification of *h*E-SLE sequence**

Using the PCR amplification procedure described in section 2.2.2.2, mastermix (10 µl) was prepared according to Table 2.16 and the amplification was done with the program shown in Table 2.17. After 1 min initial denaturation at 96°C, the amplification of the *h*E-SLE sequence was run for 25 cycles of 10 seconds denaturation at 96°C, 5 seconds annealing at 50 ˚C and 4 min extension at 60°C.

Table 2.16. Composition of mastermix for 10 µl PCR sequencing reactions.

	Mastermix
Template DNA (200-400 ng)	5 µl
Primer (10 µM)	1 µl
Terminator Ready Reaction Mix	3 µl
Millipore water	1 µl
Total	10 µl

Table 2.17. Program of PCR amplification for sequencing with the thermal ramp is 1˚C per second.

	Temperature	**Time**	**Cycle**
Initial denaturation	96 ˚C	1 min	1
Denaturation	96 ˚C	10 sec	25
Annealing	50 ˚C	5 sec	25
Extension	60 ˚C	4 min	25
Storage	4 ˚C	∞	

- **Purification of the extension products**

The amplified PCR products were transferred into a fresh 0.5 ml eppendorf tube. 10 µl of water, 2 µl of EDTA (125 mM, pH 8.0), 2 µl of sodium acetate (3 M, pH 4.6) and 50 µl of 100% ethanol were then added into the tube. After mixing and incubation on ice for 25 min, centrifugation was performed at 10000 rpm, 4 ˚C for 20 min. The supernatant was removed with 200 µl pipette tips as much as possible. After washing with 70% ethanol and centrifugation at 10000 rpm, 4˚C for 5min, the DNA pellet was air dried for 30 min at room temperature in the dark and stored at 4 ˚C in the dark until sequencing.

Sequences of the *h*E-SLE fragment were verified by alignment with the DNA sequence extracted from Swiss-prot using the Blast program bl2seq of NCBI server.

2.3.3 Construction of the plasmid pYJ-EG

2.3.3.1 Construction of the plasmid pYJ-IgG

(1) PCR-amplification of the human IgG1 tag

A part of CH_1 domain, complete CH_2 and CH_3 domains of human IgG1 Fc fragment (*h*IgG1 tag) were amplified from the genomic DNA isolated from CHO-E-sel/IgG cells with IgG1 and IgG2 primers (Figure2.8). The forward primer IgG1 contains an *Eco*R I restriction endonuclease site and the backward primer IgG2 contains an *Xho* I site and a stop codon.

Primer IgG1: 5′- CG**GAATTC**TGTGAAGTGGACAAGAAAGTT-3′
*Eco*R I
Primer IgG2: 5′- CCG**CTCGAG**TTATTTACCCGGAGACAGGGA-3′
Xho I

Figure 2.8. The forward primer IgG1 and the backward primer IgG2 designed for amplification of the *h*IgG1 tag.

The PCR amplification was done using the same procedure described in section 2.2.2.2 with the annealing temperature at 56 ˚C. The PCR products were analyzed by agarose-gel electrophoresis and purified with GenPCR purification Kit (Sigma).

(2) Restriction enzyme digestion

Restriction digestions of the PCR amplified *h*IgG1 fragment and the plasmid pcDNA3.1(+) were performed with *Xho* I and *EcoR* I at 37˚C for 3 h with Table 2.18.

Table 2.18. Composition of double digestion of the *h*IgG1 tag fragment and the plasmid pcDNA3.1(+) with *Xho* I and *EcoR* I in NEBuffer-EcoRI. Both enzymes are 100% active in this buffer.

	E-SLecEGF fragment	**pcDNA3.1(+)**
DNA fragment	30 µl	15 µl
10xNEBuffer-EcoRI	5 µl	5 µl
***Xho* I (20 U/µl)**	1 µl	1 µl
***EcoR* I (20 U/µl)**	1 µl	1 µl
BSA (1 mg/ml)	0.5 µl	0.5 µl
Sterilized water	12.5 µl	27.5 µl
Total	50 µl	50 µl

The digested *h*IgG1 tag fragments and the plasmid pcDNA3.1(+) were analyzed by agarose-gel electrophoresis and purified by agarose-gel extraction with the GenElute^{TM} Gel Purification kit (Sigma).

(3) Ligation of human IgG1 tag fragment into the pcDNA3.1(+)

The ligation of double-digested human IgG1 fragment into the same digested vector pcDNA3.1 (+) was performed using T4 DNA ligase as described in section 2.2.2.2.

(4) Transformation and isolation of the pYJ-IgG

The transformation of the plasmid pYJ-IgG into *E.coli* DH 5α competent cells and isolation of this plasmid from transformants were conducted as described in section 2.2.2.2. A number of transformed clones were then analyzed as described below.

(5) Analysis of *E.coli* transformants

- **PCR analysis**

Using IgG1 and IgG2 primers (Figure 2.8) and Taq DNA polymerase, colony PCR of *E.coli* transformants was performed as described in section 2.2.2.2.

- **Plasmid DNA sequence verification**

The plasmid pYJ-IgG was sequenced with the primer T7 (5'- TAATACGACTCACTATAGG -3') for the forward sequencing by the Biocenter sequencing group as described in section 2.3.2.6**.** The sequences of the human IgG1 tag were verified by the alignment with the DNA sequence extracted from Swiss-prot using the Blast program bl2seq of NCBI server.

2.3.3.2 Cloning of *h*E-LecEGF into the plasmid pYJ-IgG

(1) PCR amplification of the *h*E-SLE fragment

The forward primer E1 containing a *Bam*H I restriction site (Figure 2.7) and the backward primer E3 containing an *EcoR* I site and an enterokinase cleavage site (DDDDK) (Figure 2.9) were designed for amplification of the *h*E-SLE fragment from the genomic DNA of CHO-E-sel/IgG cells.

Primer E3: 5′- CG**GAATTC**CTTGTCGTCATCGTCCACAATTTGCTCACACTTGA - 3′
*Eco*R I (D D D D K)
Enterokinase recognition sequence

Figure 2.9. The backward primer E3 designed for amplification of the *h*E-SLE fragment.

The PCR amplification was performed with the annealing temperature at 56 ˚C using the procedure described in section 2.2.2.2. The amplified PCR products were analyzed by agarose-gel electrophoresis and purified with GenPCR purification Kit (Sigma).

(2) Restriction enzyme digestion

BamH I and *EcoR* I restriction endonucleases were used to double-digest the PCR amplified *h*E-SLE fragment and the vector pYJ-IgG at 37˚C for 3 h according to Table 2.19.

Table 2.19. Composition of double digestions of the PCR amplified *h*E-SLE fragment and the plasmid pYJ-IgG with *BamH* I and *EcoR* I in NEBuffer-EcoRI. Both enzymes are 100% active in this buffer.

	***h*E-SLE fragment**	**pYJG**
DNA fragment	30 µl	15 µl
10xNEBuffer-EcoRI	5 µl	5 µl
***BamH* I (20 U/µl)**	1 µl	1 µl
***EcoR* I (20 U/µl)**	1 µl	1 µl
BSA (1 mg/ml)	0.5 µl	0.5 µl
Sterilized water	12.5 µl	27.5 µl
Total	50 µl	50 µl

The digested PCR amplified *h*E-SLE fragment and the plasmid pYJ-IgG were analyzed by agarose-gel electrophoresis and purified by agarose-gel extraction with the GenElute^{TM} Gel Purification kit (Sigma).

(3) Ligation of *h*E-SLE fragment into plasmid pYJ-IgG

Using the ligation procedure described in section 2.2.2.2, the doubly digested *h*E-SLE fragments were inserted into the same digested vector pYJ-IgG using T4 DNA ligase.

(4) Transformation and isolation of plasmid pYJEG

The plasmid pYJEG was amplified by transformation of *E.coli* cloning strain DH5α and purified with GenElute Plasmid Miniprep kit (Sigma) as described in section 2.2.2.2.

(5) Analysis of *E.coli* transformants

- **PCR analysis**

Colony PCR of *E.coli* transformants was performed as described in section 2.2.2.2, using E1 (Figure 2.7) and E3 (Figure 2.9) primers and Taq DNA polymerase.

- **Plasmid DNA sequence verification**

The plasmid pYJEG was sequenced and verified by using the primer T7 (5'-TAATACGACTCACTATAGG -3') for the *h*E-SLE fragment and the Primer IgG1 (Figure 2.8) for the human IgG1 tag as described in section 2.3.2.6.

2.3.4 Transfection

2.3.4.1 Cell culture of CHO K1 cells

(1) Recovering cells from liquid nitrogen

CHO K1 cells were recovered from liquid nitrogen in complete Ham's F12 medium (Ham's F12 medium containing 10% FBS) in a T25 flask using the general method described in section 2.2.3.1.

(2) Cell splitting

When the cell monolayer reaches 100% confluent, cells were split. Trypsin-EDTA method (see section 2.2.2.3) was used to detach the cells. The collected cells were then diluted in 1:5 to1:10 with fresh Ham's F12 complete medium (Ham's F12 medium containing 10% FBS). The cells were subcultured in T75 flasks in a 37°C and 5% CO_2 humid incubator for the further transfection and protein production.

(3) Cell Freezing

CHO K1 cells were frozen in Ham's F12 medium containing 10% DMSO and 10% FBS using the method described in section 2.2.3.1.

2.3.4.2 Determination of geneticin sensitivity

In order to select the stable expression clones and subclones of the recombinant protein, the sensitivity of CHO K1 cells to geneticin was determined by the below procedure.

CHO K1 cells were seeded into a 6 well plate with approximately 25% confluent in Ham's F12 complete medium (Ham's F12 medium containing 10% FBS) and incubated at 37°C overnight. The next day, the culture medium was substituted with the medium containing varying concentrations of geneticin (0, 100, 200, 400, 600 and 800 μg/ml geneticin). The selective media were replenished every 3-4 days and the percentage of surviving cells was observed under a microscope. The number of viable cells was counted to determine the appropriate concentration of geneticin that killed cells within 2-3 weeks after addition of geneticin.

2.3.4.3 Linearization of the expression plasmids pYJE and pYJEG

The restriction endonuclease *Sca* I was used to linearize the plasmid pYJE and pYJEG. The reaction was performed at 37˚C for 3 h according to Table 2.20.

Table 2.20. Composition of digestion of the plasmids pYJE and pYJEG with *Sca* I in NEBuffer-3. *Sca* I is 100% active in this buffer.

	pYJE	**pYJEG**
DNA fragment	16 μl	16 μl
10xNEBuffer-3	5 μl	5 μl
***Sca* I (20 U/μl)**	2 μl	2 μl
Sterilized water	27 μl	27 μl
Total	50 μl	50 μl

The *Sca* I restriction endonuclease was inactivated by heating at 80˚C for 20min. The linearized plasmids were analyzed by agarose-gel electrophoresis and purified with GenPCR purification Kit (Sigma).

2.3.4.4 Transfection of the plasmids pYJE and pYJEG into CHO K1 cells

The transfection of the linearized plasmids pYJE and pYJEG were performed with Metafectene™ (Biontex)[322] by the below procedure.

CHO K1 cells were seeded into 6-well cell culture plates at $2x10^5$ cells per well in 2 ml Ham's F12 Medium containing 10% FBS. The cells were incubated at 37˚C overnight until reached 30%-50% confluency (growing area should covered 90-100%). The confluency of cells was checked under an inverted phase microscope. The solutions of DNA and metafectene transfection reagent were prepared in Polypropylene tubes as below: a) 2 μg of DNA (The expression plasmids of pYJE or pYJEG) in 100 μl OPTI-MEM I medium. b) 6 μl of metafectene in 100 μl OPTI-MEM I medium. Two solutions were then combined, mixed gently by carefully pipetting several times, and incubated at room temperature for 15-20 min in order to form the DNA-lipid complex. The DNA-lipid complex was then added to the cells and mixed

gently. In order to avoid the toxicity of the transfection mixture, the transfection mixture was removed after incubation of 3-6 hours at 37˚C and replaced with fresh medium Ham's F12 medium containing 10% FBS.

The negative control of the transfection was the cells transfected with the transfection mixture without plasmids.

2.3.5 Selection of stable expression cell lines of CHO-YJE and CHO-YJEG

500 μg/ml of geneticin was used for screening the stable expression cell lines. The concentration of geneticin was determined as described in section 2.3.4.2.

2.3.5.1 Selection of high, stable expression clones

(1) Selection of stable clones

For screening the stable clones, the transfectants were subcultured in 96-well cell culture plates in Ham's F12 selective medium (containing 10% FBS and 500 μg/ml geneticin) at 37˚C after transfection of 48 hours. The Ham's F12 selective medium was replenished to the cells every three to four days until geneticin-resistant clones were identified.

(2) Detection of high expression clones

In order to select the high expression stable clones, the expression of *h*E-LecEGF from each stable clones were detected by AP-ELISA method (see section 2.2.4.1). 100 μl of culture supernatant from each clones was analyzed in AP-ELISA assays with 10 μg/ml of mAb 7A9 as the primary antibody, a AP-labeled goat-anti-mouse antibody solution (Sigma, 1:3000 dilution) as the secondary antibody and 1 mg/ml of p-nitrophenylphosphate as the substrate. The color was developed for 15-20 min at RT prior to reading the absorbance at 405 nm in a Spectramax190 plate reader.

For the same clone, AP-ELISA assay was performed twice in order to avoid the false one. The high, stable expression clones of CHO-YJE and CHO-YJEG were then frozen in liquid nitrogen as described in section 2.3.4.1.

2.3.5.2 Selection of high, stable expression subclones

(1) Adaptation to DMEM:F12 Medium

In order to reduce the cost and facilitate the purification, the high, stable expression clones of CHO-YJE and CHO-YJEG were adapted to grow in DMEM:F12 selective medium containing 5% FBS instead of Ham's F12 selective medium containing 10% FCS prior to screening the subclones. The adaptation was performed as described in section 2.2.5.1.

(2) Selection of subclones

A limiting dilution method described by Born R.[323] was carried out to get the subclones of CHO-YJE and CHO-YJEG. The cells were diluted to 0.3 cell/well, 1 cell/well, 10 cells/well and 100 cells/ well in DMEM:F12 selective medium containing 5% FBS in 96-well cell culture plates and incubated at 37˚C in a 5% CO_2 incubator. The subclone from a single cell was inspected under the microscope.

(3) Detection of high expression subclones

The AP-ELISA was performed twice for detection of the high expression subclones of CHO-YJES and CHO-YJEGS as described in section 2.3.5.1. The high, stable expression subclones of CHO-YJES and CHO-YJEGS were then frozen in liquid nitrogen as described in section 2.3.4.1.

2.3.5.3 Characterization of subclones of CHO-YJES and CHO-YJEGS

(1) Characterization on DNA level

For the CHO-YJES subclones, the encoding gene for the signal, lectin and EGF-like domains of human E selectin (*h*E-SLE) was amplified from subclones of CHO-YJES by colony PCR with E1 and E2 primers (Figure 2.7) as described in section 2.3.2.6. For the CHO-YJEGS subclones, the PCR amplification of the *h*E-SLE fragment with E1 and E3 primers (Figure 2.7, Figure 2.9) and the human IgG1 tag with IgG1 and IgG2 primers (Figure 2.8) were performed as described in section 2.3.3.2.

(2) Characterization on protein level

- **Characterization of CHO-YJE subclones**

Supernatant preparation

The selected subclone CHO-YJES1 was cultured in T175 flasks in DMEM:F12 selective medium containing 5% FBS and 500 µg/ml geneticin for production of the *h*E-LecEGF protein. 100 ml supernatant was collected by centrifugation at 5000 rpm 10 min at 4˚C. After filtration with 0.22 µM membrane, the collected supernatant was 1:1 diluted with the binding buffer for further purification.

Purification by the mAb7A9-coupled column

A mAb 7A9 coupled column (2 ml) (see section 2.2.5.1) was used to purify *h*E-LecEGF from the supernatant of the subclone CHO-YJS culture medium (see section 2.3.5.3).

The purification was performed at 4°C on a BioLogic workstation, using the EZLogic software. The mAb 7A9 coupled column was first equilibrated with binding buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.2) for at least 10 bed volumes at the flow rate of 0.5 ml/min until the baseline was reached. The supernatant of *h*E-LecEGF was then loaded onto the column at the flow rate of 0.5 ml/min. After washing with 5-10 bed-volumes binding buffer at a flow rate of 0.5 ml/min, the *h*E-LecEGF protein was eluted isocratically with 5 bed-volumes elution buffer (100 mM glycine, 2 mM $CaCl_2$, pH 2.5) at a flow rate of 0.5-1 ml/min. 1 ml of aliquots of the eluted fractions were collected in a 1.5 ml tube containing 60 µl of Tris-HCl (1M, pH 8.8) for neutralization. The column was then neutralized by washing with 5-10 bed volumes binding buffer at a flow rate of 1 ml/min and stored in storing buffer (50 mM Tris-HCl, 1M NaCl, 2 mM $CaCl_2$, 0.025% NaN_3, pH 7.2) at 4°C for the long-term storage. The collected fractions were analyzed by 15% SDS-PAGE and pooled for further characterization.

Western blotting analysis with the anti-E-selectin N18 antibody

1-2 µg reduced and non-reduced *h*E-LecEGF protein, expressed from the CHO-YJS subclone, was analyzed by western-blotting method described in section 2.2.4.1. The anti-E-selectin N18 antibody (Santa Cruz Biotechnology) at a concentration of 2 µg/ml was employed as the primary antibody and the anti-mouse IgG (Fc specific)–alkaline phosphatase antibody (Sigma) was used as the secondary antibody. The signal detection was performed with BCIP/NBT as the substrate.

- **Characterization of the CHO-YJEG subclones**

Supernatant preparation

In order to identify the expression of *h*E-LecEGF/IgG (*h*E-LecEGF fused with C-terminal *h*IgG1 tag) from the subclone CHO-YJEGS1, 100 ml supernatant of the culture medium was prepared above.

Purification by Protein A column

The *h*E-LecEGF/IgG protein was purified with a 1 ml HiTrap rProtein A FF agarose matrix (GE Healthcare) according to the manufacturer's instructions. Using the purification procedure described in section 2.3.5.3, the purification was done using the binding buffer (20 mM sodium phosphate, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.0) for equilibrating and washing the column at the flow rate of 1 ml/min, and the elution buffer (100 mM sodium citrate, pH 3.0) at a flow rate of 1 ml/min for elution of the *h*E-LecEGF/IgG protein. The eluted protein was collected in fractions. 1 ml of aliquots of the eluted fractions was collected in a 1.5 ml tube containing 40 µl of Tris-HCl (1 M, pH 8.8) for neutralization. The column was then extensively washed with 5-10 bed volumes binding buffer at a flow rate of 1 ml/min and stored in storing buffer (20% ethanol) at 4°C for the long-term storage. The collected fractions were analyzed by 12% SDS-PAGE and pooled for further characterization.

Western-blotting analysis with the anti-human IgG1 antibody

1-2 µg reduced and non-reduced *h*E-LecEGF/IgG, purified from protein A column, was first separated by 12% SDS-PAGE. The proteins were then transferred to the nitrocellulose membrane. After blocking the nonspecific binding sites on the membrane with 3% BSA in TBS buffer, the *h*E-LecEGF/IgG protein was probed with the anti-human IgG1 monoclonal antibody conjugated with alkaline phosphatase (1 µg/ml). The detection was carried out by color development with BCIP/NBT.

2.3.6 Production of *h*E-LecEGF

The production of *h*E-LecEGF was performed by subculturing the CHO-YJES1 cells in both T-flasks and roller bottles at 37°C in the 5% CO_2 humid incubator.

2.3.6.1 Production in T-Flasks

The production of *h*E-LecEGF in T-Flasks was optimized from a protocol described by *Dragic*

Z.[324]. 1x10^6 CHO-YJES cells were seeded in 50 ml DMEM:F12 selective medium in T175 Flasks and grown to reach the full confluency in T-flasks. The production phase then started by collecting the yellowish medium and replenishing the fresh medium. The whole production phase lasted three to five weeks.

The supernatant containing the *h*E-LecEGF protein was collected after centrifugation at 5000 rpm for 10 min at 4°C. After filtration with Stericup^{TM} (0.22 μM), the supernatant was stored at 4°C or -20°C for further affinity purification.

2.3.6.2 Production in roller bottles

The production of *h*E-LecEGF in roller bottles was optimized from *Dragic Z.* [324]. 7x10^6 CHO-YJES cells were seeded in 350 ml DMEM:F12 selective medium in a roller bottle (1700cm^2, Coring) with rolling speed at 0.5 rpm for cells adhesion to the surface of the bottle and 1 rpm for cells growing. After cells reached the full confluency in the roller bottle, the production phase then started by harvesting the yellowish medium and addition of 450 ml fresh medium per bottle. The whole production phase lasted three to four weeks. The supernatant was then collected as described in section 2.3.6.1.

2.3.6.3 Sodium butyrate effect on protein expression

The effect of sodium butyrate on the protein expression was checked by the below procedure. In the production phase of the T-flask production, 1 mM, 2 mM and 4 mM of sodium butyrate were added, respectively. The culture supernatant was collected every 3 days and the production of *h*E-LecEGF was evaluated by AP-ELISA as described in section 2.3.5.1. The cell viability and cell counting was determined by Trypan blue method described in section 2.2.3.1.

2.3.7 Purification and characterization of *h*E-LecEGF expressed in CHO-K1 cells

2.3.7.1 Purification and SDS-PAGE analysis

The purification of *h*E-lecEGF expressed by CHO-YJES1 subclone was performed with a 2 ml or 5 ml mAb 7A9 coupled column as described in section 2.3.5.3. The collected fractions were analyzed by 15% SDS-PAGE (see section 2.2.4.1) and pooled for further characterization.

2.3.7.2 Deglycosylation of *h*E-LecEGF expressed in CHO-K1 cells

(1) Deglycosylation under native condition

Deglycosylation of *h*E-LecEGF (5 μg) was performed at 37°C with 1 U and 2 U of Peptide-N-glycosydase F (PNGase F, Roche) in 20 mM sodium phosphate buffer (pH 7.2) and in Tris buffer (50 mM Tris, 2 mM $CaCl_2$, pH 8.0) according to Table 2.21. After digestion for 24, 48 and 72 hours, the deglycosylated *h*E-LecEGF was analyzed on 15% SDS-PAGE under reducing condition.

Table 2.21. Composition of deglycosylation of *h*E-LecEGF under native conditions.

37°C	Incubation buffer	
	Sodium phosphate (pH 7.2)	Tris buffer (PH 8.0)
Incubation time (48, 72h)	+	+
PNGas F (1 U, 2 U)	+	+

(2) Purification of the deglycosylated *h*E-LecEGF

Ion exchange chromatography with Sepharose Q column was performed to purify the deglycosylated *h*E-LecEGF by syringe. 0.5 ml Sepharose Q Fast Flow agarose matrix (Amersham Biosciences) packed in the 1 ml Column Mobicol (MoBiTec) was first equilibrated with 5 ml Buffer I (50 mM Tris, 2 mM $CaCl_2$, pH 8.0). The protein sample in the Buffer I was then loaded onto the column drop by drop. After washing with 2.5-5 ml Buffer I, the column was washed by 2-3 ml Buffer II (50 mM Tris, 100 mM NaCl, pH 8.0) and 2-3 ml Buffer III (50 mM Tris, 500 mM NaCl, pH 8.0). 1 ml aliquots of the fractions were collected in 1.5 ml tubes. The column was then equilibrated in storing buffer (50 mM Tris, 500 mM NaCl, 20% ethanol, pH 8.0) and stored at 4°C. The collected fractions were analyzed on 15% SDS-PAGE and pooled for further characterization.

(3) Glycans detection

The GelCode Glycoprotein Staining Kit (Pierce) was used to detect the glycans of the *h*E-LecEGF and the deglycosylated *h*E-LecEGF as described in section 2.2.5.3.

2.3.7.3 Protein identification by western-blotting analysis

The *h*E-LecEGF protein (reduced and non-reduced, 1 μg) and the deglycosylated *h*E-LecEGF protein (reduced and non-reduced, 1 μg) were analyzed by western blotting method with the monoclonal anti-E-selectin N18 antibody as described in section 2.3.5.3.

2.3.7.4 Yield

The amounts of *h*E-LecEGF produced in T-flasks and roller bottles were determined by Bradford standard assay or Bradford micro-assay as described in section 2.2.4.1.

2.3.7.5 Mass spectrometry analysis

Mass spectrometry analysis was performed in Mass Spectrometry group in Biocenter, University of Basel.

(1) Peptide identification by MS

- **Sample preparation**

Pure *h*E-LecEGF (2 μg) was separated by SDS-PAGE under reducing condition and stained with Simply Blue SafeStain (invitrogen) followed by destaining with water according to the manufacturer's instructions. The protein band in the gel was excised with a razor blade and reduced with 10 mM DTT for 2 h at 37^{o}C and alkylated with iodoacetamide at 50 mM final concentration for 15 min at room temperature in the dark. The gel piece was then digested with 125 ng trypsin (Sequencing Grade, Promega) for 18 h at 37^{o}C. The peptides in the supernatant were collected and the gel piece was extracted with 0.1% formic acid/50% acetonitrile. The extract was pooled with the tryptic peptides. The digest was dried in a speed vac and redissolved in 0.1% formic acid/2% acetonitrile. 10 µl were used for mass spectrometric analysis.

- **LC-MS-MS identification**

The trypsin digested proteins were analyzed by capillary liquid chromatography tandem MS (LC/MS/MS) using a 300SB C-18 column (0.3x50mm) (Agilent Technologies, Basel, Switzerland) connected on line to an LCQ Advantage Max instrument (Thermo Finnigan, San Jose, CA, USA). A linear gradient from 2% to 60% solvent B (0.1% formic acid and 80% acetonitrile in water) in solvent A (0.1% formic acid and 2% acetonitrile in water) in 85 min was delivered with a Rheos 2000 pump (Flux Instruments, Basel, Switzerland) at a flow rate of 100 µl/min. A pre-column split was used to reduce the flow rate to approximately 500 nl/min. 10 µl sample were injected with an auto sampler. And the eluting peptides were ionized at 1.6 kV. The mass spectrometer was operated in a data-dependent fashion so that the peptide ions were automatically selected for fragmentation by collision-induced dissociation (MS/MS). The MS/MS spectra were then searched against the data bank using TurboSequest software.

(2) Molecular weight determination by MS

- **Sample preparation**

The *h*E-LecEGF protein purified from the mAb 7A9 coupled column was in the buffer containing 100 mM glycine, 60 mM Tris and 2 mM $CaCl_2$ (see section 2.3.5.3). In order to remove these salts, which are not suitable for MS analysis, the reverse phase high performance liquid chromatography (RP-HPLC) was performed on a Peptide / Protein C18 column. With the gradient program described in Table 2.22, *h*E-LecEGF (10 µg) was loaded onto the column in two times with the flow rate of 1 ml/min using buffer A (0.1% TFA in HPLC grade water) and buffer B (0.1% TFA in HPLC grade acetonitrile). The collected protein fractions were shock-frozen in liquid nitrogen, lyophilized overnight and analyzed by MS.

Table 2.22. Gradient program used for RP-HPLC purification of *h*E-LecEGF.

Time (min)	A%	B%	Flow rate(ml/min)
0	75	25	1
10	62	38	1
15	62	38	1
20	75	25	1
25	75	25	1

- **MS measurement**

The proteins were analyzed using the procedure described in LC-MS-MS identification (see above). 10 µl of sample were injected with an auto sampler. The mass spectrometer was operated in MS mode only and was scanned between 400 and 1600 *m/z* range.

2.3.7.6 Activity determination

Western-blotting, ELISA, Polymer assay and NMR methods were used to characterize the activities of pure *h*E-LecEGF and deglycosylated *h*E-LecEGF.

(1) Western-blotting with the mAb 7A9

The pure *h*E-LecEGF and the deglycosylated *h*E-LecEGF (1-2 µg) were identified by western blotting with the mAb 7A9 under native condition as described in section 2.2.5.3.

(2) ELISA

The pure *h*E-LecEGF and the deglycosylated *h*E-LecEGF (3-5 µg) were analyzed by HRP-ELISA with the mAb 7A9 as described in section 2.3.5.1.

(3) Polymer assay

The binding of sialyl Lewis-a (sLe^a) polymer to the purified *h*E-LecEGF protein was tested in a sLe^a-polymer assay[324, 325].

• Preparation of sLe^a-polymer-biotin-streptavidin-POD complex

The biotinylated sLe^a-polyacrylamide polymer (Glycotech) was dissolved in HAB buffer (20 mM Hepes, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.4) at 1 mg/ml and stored at -20°C for the long-term storage. In order to form SLe^a-polymer-biotin-streptavidin-POD complex, the matrix was prepared in a 1.5 ml tube with streptavidin-POD-conjugate (500 U/ml, Roche) according to Table 2.23. After incubation at 37°C for 2h, The sLe^a-polymer-biotin-streptavidin-POD complex (100 µg/ml) was formed. It was further stored at 4°C in the dark and it was stable over several weeks.

Table 2.23. Composition of sLe^a polymer complex.

	Matrix
sLe^a-polymer	**20 µl**
Streptavidin-POD conjugate	**80 µl**
Fetal calb serum	**20 µl**
HAB	**80 µl**
Total	**200µl**

• sLe^a Polymer assay

100 µl of *h*E-LecEGF (3 µg/ml) in HAB buffer (20 mM Hepes, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.4) were coated onto MaxiSorb 96 well ELISA plates (Nunc) at 4°C, overnight. The plates were then washed gently with HAB buffer for three times and the plates were tapped dry on the paper towels. The non-specific sites were blocked with 200 µl /well blocking buffer (3% BSA in HAB buffer) for 3 h at room temperature. After washing three times with 200 µl /well HAB buffer and tapping dry on paper towels, 100 µl /well sLe^a – polymer complex (0.1 µg/ml in HAB-buffer) were distributed in triplicate and incubated on the shaker with 56 rpm at room temperature for 3h. The plates were then washed three times with 200 µl /well HAB buffer and tapped dry on paper towels before addition of 100 µl /well ABTS solution (Bio-Rad). After the incubation with ABTS solution for 10 min in the dark, the color development was stopped with 100 µl/well oxalic acid (2%) and the absorbance was read at 415 nm in a Spectramax190 plate reader. The evaporation in the assay was prevented by incubation in a humidified chamber during assay. In the assay, the negative control used is HAB buffer and the positive control is the E-selectin/IgG protein.

• Optimization of sLe^a Polymer assay

The signal of the *h*E-LecEGF protein in sLe^a Polymer assay[324, 325] was difficult to be detected with above conditions. Hence, the sLe^a Polymer assay was optimized by the below parameters:

a. Concentration of hE-LecEGF

3 µg/ml, 5µg/ml, 10 µg/ml and 20 µg/ml of *h*E-LecEGF were used to increase the assay signal.

b. Concentration of the sLe^a polymer complex

0.1 µg/ml, 0.3 µg/ml, 0.5 µg/ml and 1 µg/ml of the sLe^a-polymer complex were employed respectively in the assay.

c. Binding buffer

HAB buffer and HAB20 buffer (HAB buffer containing 20 mM $CaCl_2$) were used.

d. Developing time

The developing time was prolonged to 10 min, 15 min and 20 min in order to increase the detection signal.

(4) NMR analysis

Two independent NMR experiments were preformed to assess the binding of CGP69669 (an antagonist of E-selectin) to *h*E-LecEGF. The T1rho experiment[326] and the STD NMR experiment[327] are subject to independent sources of experimental errors. Thus, a positive identification of ligand binding from both experiments would serve as cross-validation of the results. NMR experiments performed by Dr. Brain Cutting (Institute of molecular pharmacy, university of Basel) were conducted on a Bruker Avance DMX-500 (500 MHz) spectrometer.

• Sample preparation

Using the Amicon ultra-4 (5KDa) centrifugal filter devices and Centricon™ filtration units, the pure *h*E-LecEGF protein was concentrated and changed to the NMR assay buffer (20 mM d11-Tris, 20mM $CaCl_2$ in 99.8% D_2O, pH7.4) according to the manufacturer's instruction. 52 µM of *h*E-LecEGF in 280 µl NMR assay buffer was used for the below NMR experiments.

- **STD NMR experiment**

STD measures the transfer of magnetization to a bound ligand following irradiation of the protein. Ligands that bind protein will give rise to a signal in the STD spectrum, while non-binding ligands, as well as the proteins, will not. STD NMR experiment of *h*E-LecEGF (52 µM) with the ligand CGP69669 (1.5 mM) was measured at 200 µl using a restricted volume Shigemi NMR tube[328]. The NMR experiment was performed as described by Mayer M.[327], with the exception that the protein was saturated with a train of 40 E-Burp-1 pulses[329] of 40 Hz root-mean-square intensity for 50 ms at 0 ppm[330].

- **T1rho experiment**

To assess the binding, the transverse relaxation rates were measured. The measurement used an in-house sequence to quantitate T1rho, which is a measure of the transverse relaxation and is less sensitive to magnetic field inhomogeneity. The measurement of T1rho was advantageous with respect to spin-echo schemes, since changing from the free ligand to the ligand complexed with the protein might have different levels of magnetic field homogeneity.

T1rho experiment of *h*E-LecEGF with the ligand CGP69669 was also measured at 200 µl using Shigemi NMR tube. The intensity of 1.5 mM ligands with or without protein (52 µM) was measured at the time of 0.01, 0.05, 0.1, 0.15 and 0.2 seconds. The signal loss with the increasing relaxation times was quantified and analyzed with the Prism curve-fitting software package from GraphPad Software, Inc.

2.4 Capture-binding assay development

A capture-binding assay was created based on the references[25,36,325] to evaluate the binding affinity of the *h*E-selectin ligands to *h*E-LecEGF. This assay can evaluate the binding activity of the purified *h*E-LecEGF as well.

2.4.1 Selection of Anti-E selectin antibody

The appropriate anti-E-selectin antibody was searched for immobilization of the *h*E-LecEGF protein on ELISA plates from the literature[25].

2.4.2 Capture-binding assay

The capture-binding assay was developed from the references[36,325].

100 µl/well of anti-E-selectin/anti-P-selectin antibody BBA1 in the coating buffer (50 mM Na_2CO_3, pH 9.6) was coated onto MaxiSorb 96 well ELISA plates (Nunc) at 4°C, overnight. After washing three times with 200 µl/well HAB20 buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 20 mM $CaCl_2$), the plates were tapped dry on the paper towels. The non-specific sites were blocked with 200 µl/well blocking buffer (3% BSA in HAB20 buffer) for 3 h at room temperature. After washing three times with 200 µl/well HAB20 buffer and tapping dry on paper towels, 100 µl/well *h*E-LecEGF in HAB20 buffer were added and incubated at 4°C and overnight. After three times washing with 200 µl/well HAB20 buffer, 100 µl/well sLe^a-polymer complex at a appropriate concentration were distributed in triplicate and incubated on the shaker with 56 rpm or 250 rpm at room temperature for 3 h. The plates were then washed three times with 200 µl/well HAB20 buffer and tapped dry on paper towels. After addition of 100 µl/well ABTS solution (Bio-Rad), the plates were incubated for 10 min in the dark. The color development was stopped with 100 µl /well oxalic acid (2%) and the absorbance was read at 415 nm in a Spectramax190 plate reader. The evaporation in the assay was prevented by incubation in a humidified chamber during assay. The negative control used in the assay is the anti-E-selectin/anti-P-selectin antibody BBA1 and the positive control is E-selectin/IgG protein.

2.4.3 Optimization of capture-binding assay

The capture-binding assay was further optimized in different concentrations of *h*E-LecEGF (1 µg/ml, 3 µg/ml and 5 µg/ml) and different concentrations of the sLe^a polymer complex (0.1 µg/ml, 0.3 µg/ml and 0.5 µg/ml).

2.4.4 EC_{50} determination of sLe^a-polymer

The EC_{50} of the sLe^a-polymer complex to the protein *h*E-LecEGF was determined by the capture-binding assay with serial concentrations of sLe^a-polymer complex (0.3 ng/ml-300 ng/ml) as described in section 2.4.2. The value of EC_{50} of sLe^a-polymer complex to *h*E-LecEGF was calculated using the Prism4 software.

2.4.5 Competitive capture-binding assay

Using the procedure described in section 2.4.2, the competitive capture-binding assay was performed by adding 50 µl of E-selectin ligands in serial concentrations and 50 µl of 0.1 µg/ml sLe^a-polymer complex simultaneously instead of addition of 100 µl/well sLe^a-polymer complex (0.1 µg/ml) in the capture binding assay. The developing time in the competitive capture-binding assay is around 5 min. Using the Prism4 software, the binding curve of E-selectin antagonist was drawn and the IC_{50} of antagonist to *h*E-LecEGF was calculated.

2.4.6 Surface plasmon resonance assay

Surface plasmon resonance (Biacore) assays performed by Celine Weckerle (Institute of molecular pharmacy, university of Basel) were measured on a Biacore 3000 instrument (GE Healthcare, Freiburg, Germany) at 25 °C. The running buffer was HBS-P buffer (10 mM HEPES, 150 mM NaCl, pH 7.4, 0.002% v/v surfactant P20). For all experiments, a polyclonal goat anti-human Fc specific antibody (Sigma) was first immobilized onto the research grade CM5 sensor chip via amine coupling using the manufacturer's protocol (Biacore). In a typical experiment, a 20 µg/ml solution of the polyclonal antibody diluted in acetate buffer (10 mM sodium acetate, pH 5) was injected at 10 µl/min for 10 min over all flow cells. Following the polyclonal antibody coupling, the flow path was changed to exclude the reference flow cell containing only immobilized polyclonal antibody. A 50 µg/ml solution of E-Selectin/IgG diluted in acetate buffer (10 mM sodium acetate, pH 5.5) was injected at 5 µl/min for 20 min over a single flow cell, designated the active flow cell. The reference and active flow cells were

equilibrated at 5 µl/min for 12 h in HBS-P running buffer that was supplemented with 20 mM calcium. Before injecting the analyte, the system was equilibrated for 2 h in HBS-P running buffer that was supplemented with 20 mM calcium and 2.5% DMSO (v/v). Different concentrations of analyte were prepared in HBS-P buffer that was supplemented with 20 mM calcium and 2.5% DMSO (v/v). The analyte dilutions were injected with a 60 s dissociation time over the reference and the active flow cells. To compare the binding affinity of the different selectin ligands, the sensorgrams from SPR experiments were processed with Scrubber-2.0a (BioLogic Software Pty Ltd., Campbell, Australia). The response observed in the reference flow cell was subtracted from the active flow cell. The apparent equilibrium dissociation constant KD was determined by using a simple steady state affinity 1:1 binding model. Because of the influence of DMSO to the binding signal, a calibration was necessary[331]. For that purpose, different solutions of DMSO were injected before the each cycle of measurement. The signal corrections based on the calibration solutions were directly performed during binding evaluation in the software Scrubber 2.0a.

2.5 Crystallization of *h*E-LecEGF

Crystallization of *h*E-LecEGF was performed by a sitting-drop method on the EMBO course[332] in OPPF, University of Oxford.

2.5.1 Sample preparation

Pure *h*E-LecEGF was concentrated to a final concentration of ~ 9 mg/ml in a ~ 60 µl Tris buffer (60 mM Tris, 2 mM $CaCl_2$) by ultracentrifugation.

2.5.2 Crystallization

The crystallization screening of *h*E-LecEGF was performed with Hampton crystallization screening kit I and II at 4°C and 20°C using the nanoliter-scale sitting-drop vapour-diffusion method. Three plates were done by Cartesian instrument in the single-dispense mode. The droplet for crystallization was composed of 100 nl of protein plus 100 nl of reservoir solution. The crystallization was performed with standard crystallization screening kits (Hampton) on the 96 well crystallization plates (Greiner). The barcoded crystallization plates were entered into an automated storage system with a fully integrated imaging system. The plates were imaged in accordance with a pre-programmed schedule and the resulting digital data for each droplet are harvested into a laboratory information management system, scored by crystal recognition software and displayed for analysis via a web-based interface.

3. Results and Discussion

3.1 Sequence analysis

3.1.1 DNA sequence

The amino acid sequence of human E-selectin was extracted from UniProtKB/Swiss-Prot with the entry number P16581 and used for sequence analysis. The nucleic acid sequence of human E-selectin was extracted from GenBank with the entry number M30640 and used for primers design and DNA sequencing analysis. The amino acid and nucleic acid sequences of human E-selectin are shown in Figure 3.1. The domains of human E-selectin are also shown in Figure 3.1, including the N-terminal signal sequence (21 amino acids), the lectin domain (118 amino acids), the EGF-like domain (36 amino acids), 6 consensus repeat domains, the transmembrane region (22 amino acids) and the C-terminal cytoplasmic domain (32 amino acids).

```
N:  atgattgcttcacagtttctctcagctctcactttggtgcttctcattaaagagagtgga
A:  M··I··A··S··Q··F··L··S··A··L··T··L··V··L··L··I··K··E··S··G··   20

N:  gcctggtcttacaacacctccacggaagctatgacttatgatgaggccagtgcttattgt
A:  A··W··S··Y··N··T··S··T··E··A··M··T··Y··D··E··A··S··A··Y··C··   40

N:  cagcaaaggtacacacacctggttgcaattcaaaacaaagaagagattgagtacctaaac
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N:  tccatattgagctattcaccaagttattactggattggaatcagaaaagtcaacaatgtg
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N:  tgggtctgggtaggaacccagaaacctctgacagaagaagccaagaactgggctccaggt
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N:  gaacccaacaataggcaaaaagatgaggactgcgtggagatctacatcaagagagaaaaa
A:  E··P··N··N··R··Q··K··D··E··D··C··V··E··I··Y··I··K··R··E··K··  120

N:  gatgtgggcatgtggaatgatgagaggtgcagcaagaagaagcttgccctatgctacaca
A:  D··V··G··M··W··N··D··E··R··C··S··K··K··K··L··A··L··C··Y··T··  140

N:  gctgcctgtaccaatacatcctgcagtggccacggtgaatgtgtagagaccatcaataat
A:  A··A··C··T··N··T··S··C··S··G··H··G··E··C··V··E··T··I··N··N··  160

N:  tacacttgcaagtgtgaccctggcttcagtggactcaagtgtgagcaaattgtgaactgt
A:  Y··T··C··K··C··D··P··G··F··S··G··L··K··C··E··Q··I··V··N··C··  180

N:  acagccctggaatcccctgagcatggaagcctggtttgcagtcacccactgggaaacttc
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N:  agctacaattcttcctgctctatcagctgtgataggggttacctgccaagcagcatggag
A:  S··Y··N··S··S··C··S··I··S··C··D··R··G··Y··L··P··S··S··M··E··  220

N:  accatgcagtgtatgtcctctggagaatggagtgctcctattccagcctgcaatgtggtt
A:  T··M··Q··C··M··S··S··G··E··W··S··A··P··I··P··A··C··N··V··V··  240
```

```
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A:  E··C··D··A··V··T··N··P··A··N··G··F··V··E··C··F··Q··N··P··G··  260

N:  agcttcccatggaacacaacctgtacatttgactgtgaagaaggatttgaactaatggga
A:  S··F··P··W··N··T··T··C··T··F··D··C··E··E··G··F··E··L··M··G··  280

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A:  A··Q··S··L··Q··C··T··S··S··G··N··W··D··N··E··K··P··T··C··K··  300

N:  gctgtgacatgcagggccgtccgccagcctcagaatggctctgtgaggtgcagccattcc
A:  A··V··T··C··R··A··V··R··Q··P··Q··N··G··S··V··R··C··S··H··S··  320

N:  cctgctggagagttcaccttcaaatcatcctgcaacttcacctgtgaggaaggcttcatg
A:  P··A··G··E··F··T··F··K··S··S··C··N··F··T··C··E··E··G··F··M··  340

N: ttgcagggaccagcccaggttgaatgcaccactcaagggcagtggacacagcaaatccca
A:  L··Q··G··P··A··Q··V··E··C··T··T··Q··G··Q··W··T··Q··Q··I··P··  360

N:  gtttgtgaagctttccagtgcacagccttgtccaaccccgagcgaggctacatgaattgt
A:  V··C··E··A··F··Q··C··T··A··L··S··N··P··E··R··G··Y··M··N··C··  380

N:  cttcctagtgcttctggcagtttccgttatgggtccagctgtgagttctcctgtgagcag
A:  L··P··S··A··S··G··S··F··R··Y··G··S··S··C··E··F··S··C··E··Q··  400

N:  ggttttgtgttgaagggatccaaaaggctccaatgtggccccacaggggagtgggacaac
A:  G··F··V··L··K··G··S··K··R··L··Q··C··G··P··T··G··E··W··D··N··  420

N:  gagaagcccacatgtgaagctgtgagatgcgatgctgtccaccagcccccgaagggtttg
A:  E··K··P··T··C··E··A··V··R··C··D··A··V··H··Q··P··P··K··G··L··  440

N: gtgaggtgtgctcattcccctattggagaattcacctacaagtcctcttgtgccttcagc
A:  V··R··C··A··H··S··P··I··G··E··F··T··Y··K··S··S··C··A··F··S··  460

N:  tgtgaggagggatttgaattacatggatcaactcaacttgagtgcacatctcagggacaa
A:  C··E··E··G··F··E··L··H··G··S··T··Q··L··E··C··T··S··Q··G··Q··  480

N:  tggacagaagaggttccttcctgccaagtggtaaaatgttcaagcctggcagttccggga
A:  W··T··E··E··V··P··S··C··Q··V··V··K··C··S··S··L··A··V··P··G··  500

N:  aagatcaacatgagctgcagtggggagcccgtgtttggcactgtgtgcaagttcgcctgt
A:  K··I··N··M··S··C··S··G··E··P··V··F··G··T··V··C··K··F··A··C··  520

N:  cctgaaggatggacgctcaatggctctgcagctcggacatgtggagccacaggacactgg
A:  P··E··G··W··T··L··N··G··S··A··A··R··T··C··G··A··T··G··H··W··  540

N:  tctggcctgctacctacctgtgaagctcccactgagtccaacattcccttggtagctgga
A:  S··G··L··L··P··T··C··E··A··P··T··E··S··N··I··P··L··V··A··G··  560

N:  ctttctgctgctggactctccctcctgacattagcaccatttctcctctggcttcggaaa
A:  L··S··A··A··G··L··S··L··L··T··L··A··P··F··L··L··W··L··R··K··  580

N:  tgcttacggaaagcaaagaaatttgttcctgccagcagctgccaaagccttgaatcagat
A:  C··L··R··K··A··K··K··F··V··P··A··S··S··C··Q··S··L··E··S··D··  600

N:  ggaagctaccaaaagccttcttacatcctt
A:  G··S··Y··Q··K··P··S··Y··I··L··  610
```

Figure 3.1. The nucleic acid sequence (N) and amino acid sequence (A) of human E-selectin. The N-terminal signal sequence is shown in grey, the lectin domain in red, the EGF-like domain in blue, 6 consensus repeat domains in green, the transmembrane region in purple and the C-terminal cytoplasmic domain in black.

3.1.2 Prediction of post-translational modification

3.1.2.1 Prediction of N-glycosylation sites

The potential N-glycosylation site in *h*E-LecEGF was predicted by NetNglyc1.0 server as described in section 2.1.2.1. Asn4, Asn124 and Asn139 were predicted as the potential N-glycosylation sites by examining the sequence context of Asn-Xaa-Ser/Thr sequons, including Asn-Pro-Ser/Thr.

3.1.2.2 Prediction of O-glycosylation sites

The prediction of the O-glycosylation site in *h*E-LecEGF by the program of NetOglyc3.1 and OGPET showed that there is no potential O-glycosylation site in *h*E-LecEGF.

3.2 Cloning, Expression, Purification and Characterization of Human E-LecEGF in Insect Cells

The baculovirus-infected insect cell expression system was initially chosen for the expression of the lectin and EGF-like domains of human E-selectin (*h*E-LecEGF). This expression system has the advantages of high expression of the target gene, ease of scale-up and the ablity to produce proteins with post-translational modifications[261].

3.2.1 Cloning of *h*E-LecEGF

3.2.1.1 Construction of the secretion plasmid pFastBacYJS

In order to express *h*E-LecEGF in a secreted form in insect cells, the secretion plasmid pFastBacYJS was first generated. The gp67 signal sequence, the most effective baculovirus-encoded signal sequence for protein secretion in insect cells[261], was introduced to the pFastBac^{TM}1 vector to construct the plasmid pFastBacYJS. pFastBac^{TM}1 contains the strong promoter of polyhedrin promoter for the expression of the recombinant protein in insect cells (see Figure 2.3). Thus, the construct pFastBacYJS secretes the proteins of interest under the control of polyhedrin promoter in insect cells system.

(1) Cloning of the gp67 signal sequence

The gp67 signal sequence (114 bp) was amplified by PCR from the plasmid pAcGP67-A (9761 bp) baculovirus transfer vector with 5' primer SN1 containing a *Bam*H I restriction endonuclease site and 3' primer SN2 containing an *Xba* I restriction site as described in section 2.2.2.2. The PCR product was then analyzed on 2% agarose gel (Figure 3.2). In order to construct the plasmid pFastBacYJS, a double-digested PCR fragment of the gp67 signal sequence with *BamH* I and *Xba* I restriction endonucleases was ligated by T4 DNA ligase into the insect cell expression vector pFastBac^{TM}1, which was also double-digested with these two restriction endonucleases. After transformation into *E.coli* DH5α and clone screening, the constructs pFastBacYJS (4824 bp) (Figure 3.3) were purified using the GenElute Plasmid Miniprep kit (Sigma) and were further characterized.

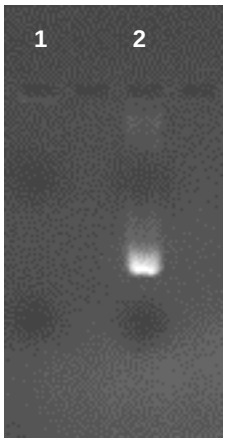


Figure 3.2. PCR amplified fragment of the gp67 signal sequence on 2% agarose gel stained with ethidium bromide. Lane 1: Negative control; Lane 2: The gp67 signal sequence amplified with SN1 and SN2 primers and pfu DNA polymerase.

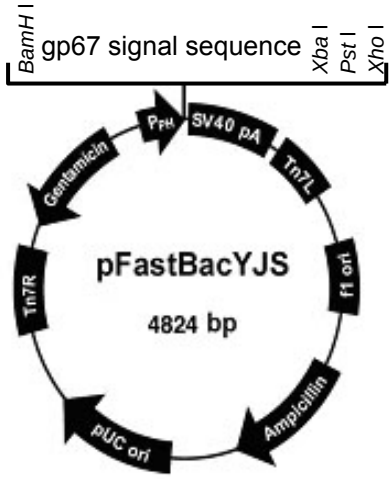


Figure 3.3. Map of the plasmid pFastBacYJS.

(2) Characterization of the plasmid pFastBacYJS

Colony PCR, restriction enzyme digestion and DNA sequencing were performed to confirm that the pFastBacYJS constructs contain the correct gp67 signal sequence.

- **Colony PCR**

Colony PCR was performed to screen the recombinant clones by amplifying the gp67 signal sequence with SN1and SN2 primers and *Taq* DNA polymerase as described in section 2.2.2.2. The amplified fragments (133 bp) from clones C1-C8 indicated the presence of the gp67 signal sequence in the clones C2-C8 (Figure 3.4).

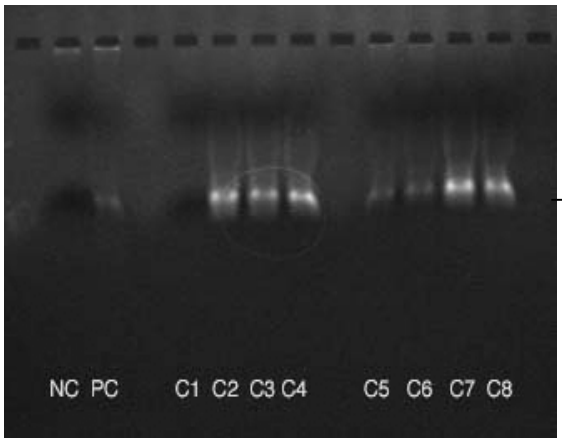


Figure 3.4. Colony PCR amplified fragments of the gp67 signal sequence on 2% agarose gel with SN1 and SN2 primers and Taq DNA polymerase. NC: Negative control; PC: Positive control with the plasmid pAcGPgp67-A; C1-C8: PCR fragments amplified from clones C1-C8.

• **Restriction enzyme digestion**

Double digestions of the pFastBacYJS constructs were performed with the restriction endonucleases *BamH* I and *Xba* I. The constructs pFastBacYJS (pFastBacYJS 2, 3, 4 and 8) were cut into the gp67 signal sequence fragments (124 bp) and pFastBac^{TM}1 vectors (4700 bp), respectively (Figure 3.5).

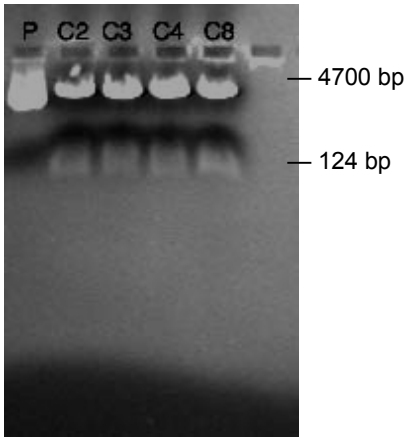


Figure 3.5. Double digestions of constructs pFastBacYJS2, 3, 4 and 8 with *BamH* I and *Xba* I on 2% agarose gel stained with ethidium bromide. P: Plasmid pFastBacYJS; C2, C3, C4, and C8: Plasmids pFastBacYJS 2, 3, 4 and 8.

• **DNA sequencing of the plasmid pFastBacYJS**

Two clones of the plasmid pFastBacYJS were sequenced with the FastBacf primer for plus strand determination as described in section 2.2.2.2. Alignment by the Blast program bl2seq showed that the 153 bases sequencing result overlapped with the gp67 signal sequence and the identity was 100% (Figure 3.6). This result indicated that both of these two constructs pFastBacYJS contained the correct gp67 signal sequence.

```
Query15  TATTNTACCGTCCCACCATCGGGCGCGGATCCATGCTACTAGTAAATCAGTCACACCAAG 74
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct1   TATTNTACCGTCCCACCATCGGGCGCGGATCCATGCTACTAGTAAATCAGTCACACCAAG 60

Query75  GCTTCAATAAGGAACACACAAGCAAGATGGTAAGCGCTATTGTTTTATATGTGCTTTTGG 114
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct61  GCTTCAATAAGGAACACACAAGCAAGATGGTAAGCGCTATTGTTTTATATGTGCTTTTGG 120

Query135CGGCGGCGGCGCATTCTGCCTTTGCGTCTAGAG 167
         |||||||||||||||||||||||||||||||
Sbjct121CGGCGGCGGCGCATTCTGCCTTTGCGTCTAGAG 153
```

Figure 3.6. Bl2seq alignment of the plus strand sequence of the pFastBacYJS sequencing result (Query) and the gp67 signal sequence (in gray) in plasmid pFastBac^{TM}1 (Sbjct). The identity was 100% (153/153residues) in the alignment.

3.2.1.2 Construction of the *h*E-LecEGF expression plasmid pFastBacYJSE

In order to express *h*E-LecEGF in insect cells, the expression plasmid pFastBacYJSE was constructed by inserting the PCR fragment of *h*E-LecEGF into the vector pFastBacYJS. An N-terminal Flag-tag was fused to the *h*E-LecEGF by primer design to facilitate the purification with affinity chromatography (Figure 3.7). The Flag-tag can be completely removed by the enterokinase to avoid interference of the tag in structure studies.

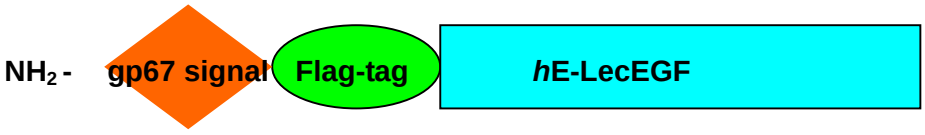


Figure 3.7. Schematic representation of *h*E-LecEGF expressed in insect cells.

(1) Genomic DNA isolation from CHO-E-sel/IgG cells

CHO-E-sel/IgG cells, generated by Novartis, are CHO cells expressing the extracellular domains (the lectin, the EGF-like and consensus repeats domains) of human E-selectin with a C-terminal human IgG1 tag[333]. The genomic DNA of CHO-E-sel/IgG cells was isolated using a WIZARD Genomic Purification Kit (Promega) as described in section 2.2.2.3, analyzed on 0.8% agarose gel (Figure 3.8) and used as the template for PCR amplification of the *h*E-LecEGF sequence.

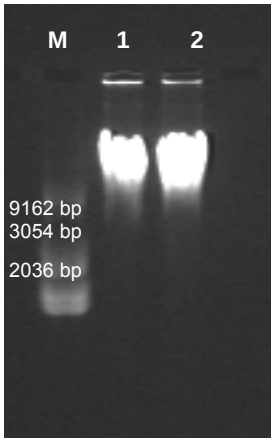


Figure 3.8. Isolated genomic DNA of CHO-E-sel-IgG cells (Lane 1, 2) on 0.8% agarose gel stained with ethidium bromide. Lane M: DNA marker X.

(2) Cloning of *h*E-LecEGF into the pFastBacYJS vector

5' primer E-FLE1 and 3' primer E-ELE2 were designed to amplify the *h*E-LecEGF sequence from the genomic DNA of CHO-E-sel/IgG cells (see section 2.2.2.3). The amplified fragments include the lectin and the EGF-like domain and three additional amino acids of the consensus repeat domain of *h*E-selectin, like the construct used previously for the crystallization of *h*E-LecEGF[30,31]. The 5' primer E-FLE1 contains an *Xba* I restriction endonuclease site and a flag tag (DYKDDDDK) designed for affinity purification and identification of the recombinant protein. Protease enterokinase can recognize the sequence DDDDK in the flag tag and cleave after the K residue to completely remove the tag. The 3' primer E-ELE2 introduces a stop codon after the *h*E-LecEGF sequence and an *Xho* I restriction endonuclease site for cloning. The lectin and the EGF-like domains of human E-selectin (471 bp) were amplified by PCR as described in section 2.2.2.3. The analysis of PCR product is shown in Figure 3.9.

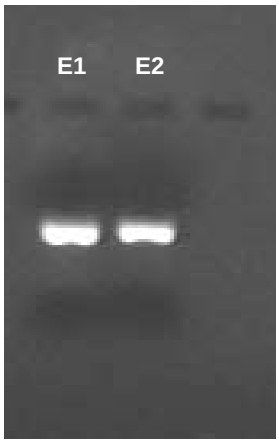


Figure 3.9. PCR products 1 and 2 of *h*E-LecEGF (Lane E1 and E2) amplified with E-LEF1 and E-LEF2 primers and *pfu* DNA polymerase on 1.5% agarose gel.

Using the same restriction/ligation cloning method for the construction of the plasmid pFastBacYJS (see section 2.2.2.2), the *h*E-LecEGF expression plasmid pFastBacYJSE (5241 bp, Figure 3.10) was constructed by insertion of the PCR products of *h*E-LecEGF into the vector pFastBacYJS (Figure 3.3). The constructed plasmid pFastBacYJSE contains the gp67 signal sequence and the *h*E-LecEGF sequence fused with an N-terminal Flag tag. As shown in Figure 3.10, the expression of *h*E-LecEGF was regulated by the polyhedrin promoter (P_{PH}).

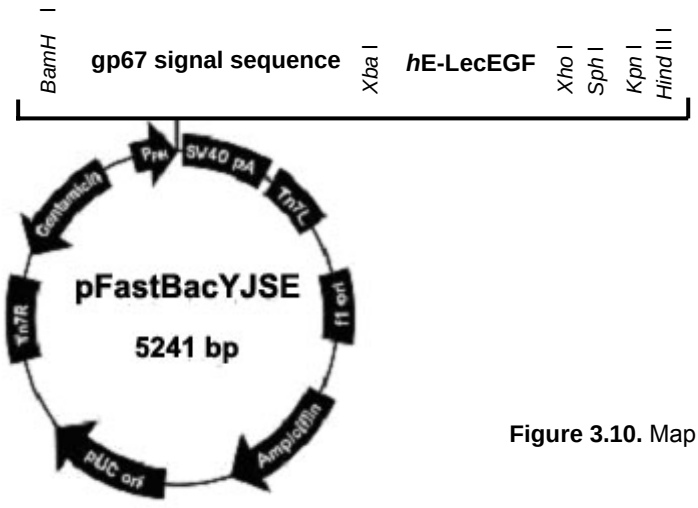


Figure 3.10. Map of the plasmid pFastBacYJSE.

(3) Characterization of the expression plasmid pFastBacYJSE

pFastBacYJSE constructs were confirmed by colony PCR, restriction enzyme digestion and DNA sequencing.

• **Colony PCR**

Colony PCR of the clones C6-C8 was performed to verify the presence of the *h*E-LecEGF sequence with E-FLE1 and E-FLE2 primers and *Taq* DNA polymerase. The amplified fragment (517 bp) from each clone (Figure 3.11) indicated the presence of the *h*E-LecEGF sequence. The positive control of colony PCR was the genomic DNA of CHO-E-sel/IgG and the negative control was the plasmid pFastBacYJS.

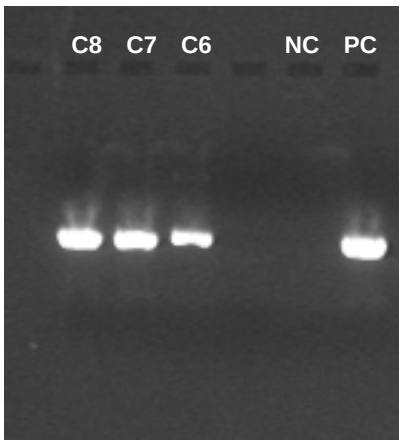


Figure 3.11. Colony PCR amplified fragments of *h*E-LecEGF on 1.5% agarose gel with E-FLE1 and E-FLE2 primers and *Taq* DNA polymerase. NC: Negative control (plasmid pFastBasYJS); PC: Positive control, the genomic DNA of CHO-E-sel-IgG cells; C6-C8: plasmids of pFastBacYJSE5-8.

• **Restriction digestion analysis**

Double digestions of pFastBacYJSE6 and pFastBacYJSE8 were performed with the restriction endonucleases *Xba* I and *Xho* I. Two fragments of the pFastBacYJS vector (4737 bp) and *h*E-LecEGF (504 bp) were generated after digestion as shown in Figure 3.12, indicating the presence of the *h*E-LecEGF sequence in the constructs.

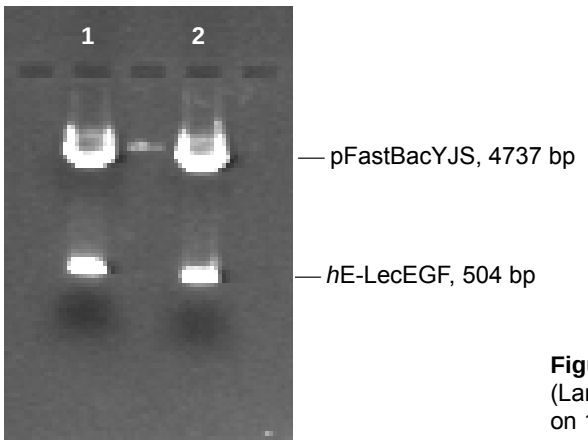


Figure 3.12. Double digestions of plasmid pFastBacYJSE6 (Lane 1) and pFastBacYJSE8 (Lane 2) with *Xba* I and *Xho* I on 1.5% agarose gel.

- **DNA sequencing of the pFastBacYJSE plasmids**

DNA sequencing of two constructs of pFastBacYJSE was performed with the primer FastBacf for plus strand determination as described in section 2.2.2.3. Alignment by the Blast program bl2seq showed that the sequence displayed 100% identity with the gp67 signal sequence (114 bp) and the *h*E-LecEGF fused with an N-terminal flag tag (498 bp) (Figure 3.13). It indicates that both of these two constructs pFastBacYJSE are correct.

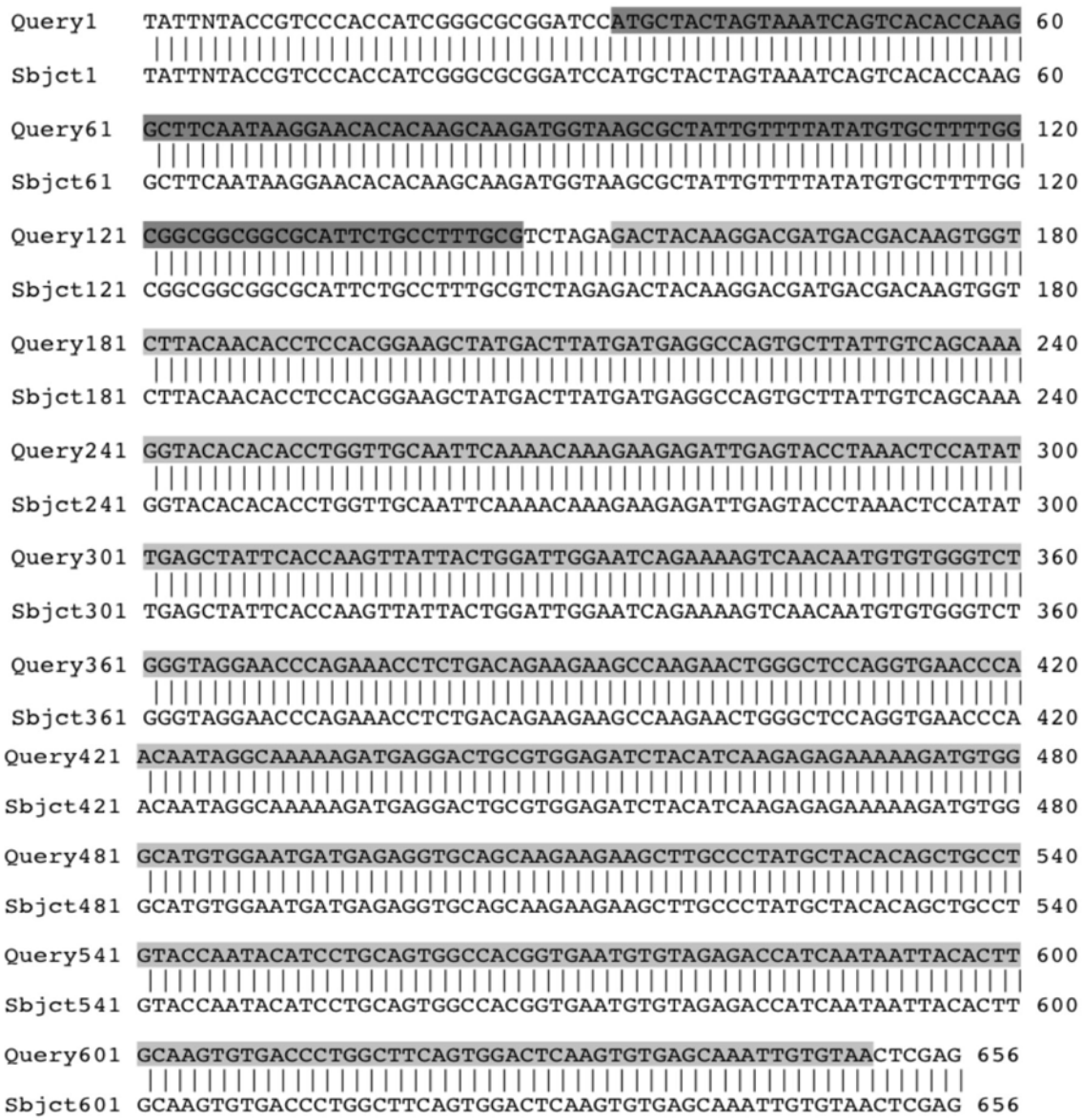


Figure 3.13. Bl2seq alignment of the plus strand sequence of the pFastBacYJSE (Query) and the gp67 signal sequence fused with the *h*E-LecEGF containing an N-terminal flag tag (Sbjct). The identity was 100%. The gp67 sequence is shown in deep grey and *h*E-LecEGF in light gray.

3.2.2 Generation of the recombinant Bacmid

Recombinant bacmids were generated by transformation of the expression plasmid pFastBacYJSE (1ng) into MAX Efficiency® DH10Bac™ competent cells (see section 2.2.2.4). The DH10Bac™ *E.coli* strain contains a helper plasmid for supplying the transposition protein and a baculovirus shuttle vector (bacmid). The recombinant bacmid was generated by transposition of the mini-Tn7 element on the pFastBac™1 constructs to the mini-attTn7 site on the bacmid with transposition protein. Five recombinant bacmids' DNA were generated and isolated. The isolated recombinant bacmid DNA is greater than 135kb in size. Since restriction enzyme analysis is difficult to perform with DNA of this size, PCR analysis was used to verify the presence of the *h*E-LecEGF gene in the recombinant bacmid. The transposed pFastBacYJSE sequence in the recombinant bacmid can be amplified by M13 Forward (-40) and M13 Reverse primers as shown in Figure 3.14.

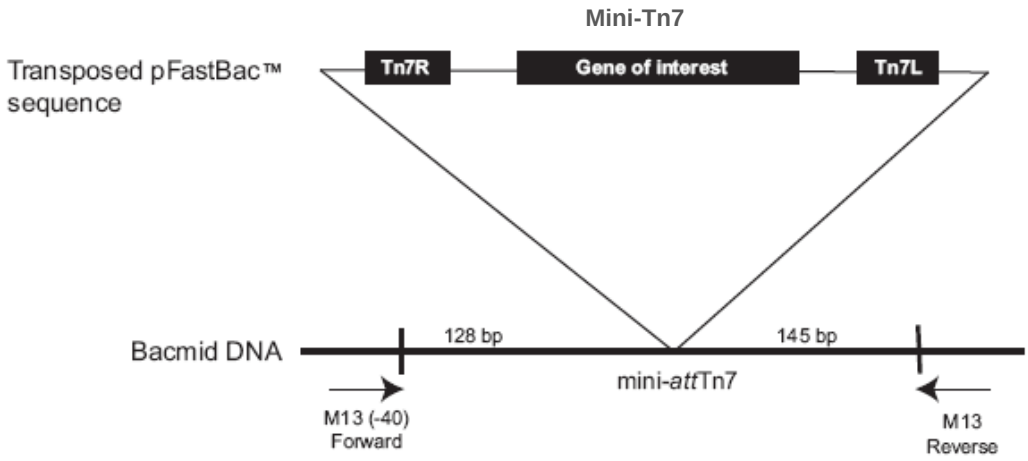


Figure 3.14. Schematic representation of the transposed sequence in bacmid DNA amplified by M13 Forward and M13 Reverse primers.

The presence of PCR fragments of 514 bp amplified with E-FLE1 (Forward) and E-FLE2 primers and the presence of PCR fragments of ~3000 bp on 1% agarose gel with M13 Forward (-40) and M13 Reverse primers indicated that the purified recombinant bacmids DNA contain the *h*E-LecEGF sequence (Figure 3.15).

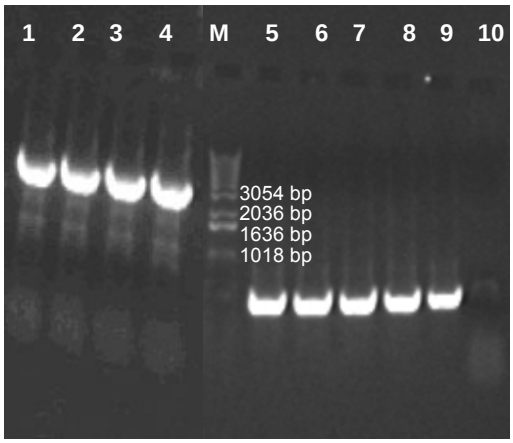


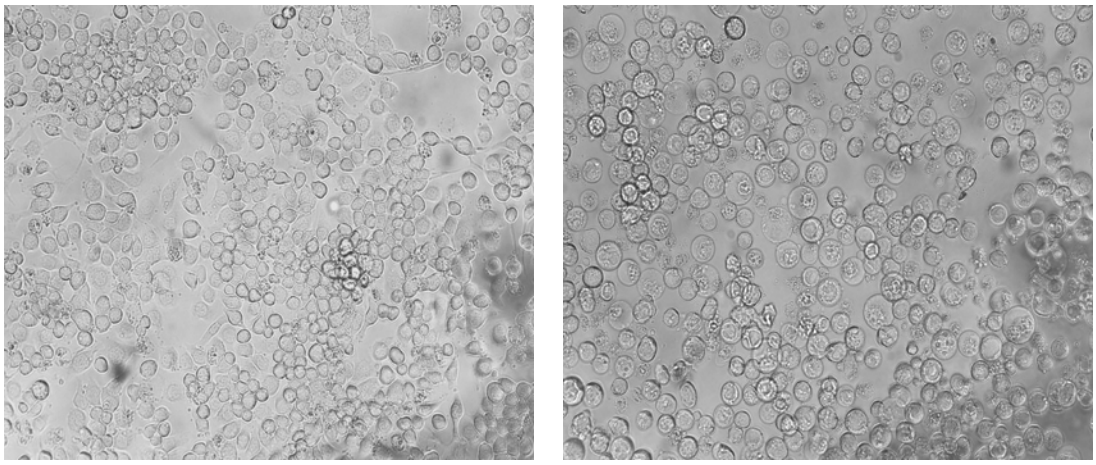
Figure 3.15. Identification of the purified recombinant bacmids1, 2, 3 and 4 by PCR amplification with *Taq* DNA polymerase on 1% agarose gel. Lane 1-4: Fragments amplified from bacmids 1-4 with M13 forward and M13 reverse primers; Lane M: DNA marker X; Lane 5-8: Fragments amplified from bacmids 1-4 with E-FLE1 and E-FLE2 primers; Lane 9: Positive control with the genomic DNA of CHO-E-sel/IgG cells; Lane 10: Negative control.

3.2.3 Production of the recombinant baculovirus

3.2.3.1 Transfection of insect cells and isolation of P1 viral stock

Transfection of insect cells Sf9 (9 x 10^5 cells, viability > 97%) with four purified recombinant Bacmids DNA (1 μg) was performed in an unsupplemented Grace's Medium in a 6-well tissue culture plate, respectively, in order to get the high expression biculovirus (see section 2.2.3.3). 6 μl of Cellfectin® Reagent, a liposome formulation, was used for mediating the transfection. After 5 hours of transfection, the cells were cultured in the complete growth media Sf-900 II SFM at 27 °C for further 72 hours or until signs of viral infection were observed by using an inverted phase microscope at 400x magnification.

P1 viral stock was isolated and collected when the cells showed signs of late and very late infection as described in section 2.2.3.4 (see Figure 3.16). Compared to the non-transfected cells, signs of the late infection are that the cell growth stops, cells increase 25-50% in cell diameter, the viral budding and vesicular appear, and cells detach from the plate. Signs of very late infection are that the cells appear lysed and show signs of clearing in the monolayer. The collected P1 viral stock containing 2% fetal bovine serum was then used for further viral plaque assay and plaque purification.



a. b.

Figure 3.16. Non-transfected Sf9 cells (a) and Sf9 cells transfected with the recombinant baculovirus of *h*E-LecEGF (b) were observed after transfection of 72 hours under the inverted phase microscope at 400x magnification.

3.2.3.2 Viral Plaque Assay

In order to determine the titer of the baculoviral stock and isolate the single viral clone, viral plaque assay was performed (see section 2.2.3.5). The titer of P1 viral stock was determined as $2x10^6$ pfu/ml and the single viral clones were isolated for further PCR identification and virus amplification. PCR amplification of the *h*E-LecEGF sequence from the isolated single viral clones $P1_1$ and $P1_2$ was performed as described in section 2.2.3.8. The amplified fragments of *h*E-LecEGF analyzed on 1.5% agarose gel indicated that the isolated single viral clones $P1_1$ and $P1_2$ contained the recombinant sequence of *h*E-LecEGF (Figure 3.17).

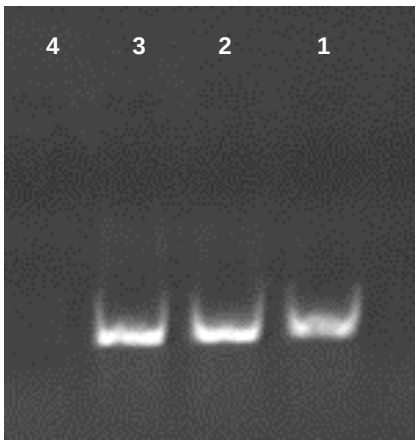


Figure 3.17. PCR amplified fragments of *h*E-LecEGF from the isolated single viral clones $P1_1$ (Lane 1) and $P1_2$ (Lane 2) on 1.5% agarose gel stained with ethidium bromide. Lane 3: Positive control with the plasmid pFastBacYJSE; Lane 4: Negative control.

3.2.3.3 Preparation of P2 and P3 viral stocks

The collected P1 viral stock is a small-scale and low-titer stock, therefore, the high-titer viral stocks P2 and P3 were amplified for further expression using P1 or P2 viral stock with an MOI of 0.05 (see section 2.2.3.6 and 2.2.3.7). The titers of P2 and P3 were determined to be ~ $2x10^7$ pfu/ml and ~ $1x10^8$ pfu/ml, respectively, by viral plaque assay.

3.2.4 Expression of the recombinant protein *h*E-LecEGF

The expression condition of the recombinant protein *h*E-LecEGF was optimized by using different cell lines, cell density, MOI and time course (see section 2.2.4.2).

Sf9 and High Five™ cells were selected for secretion of *h*E-LecEGF with an MOI of 1, 2, 5 and10, and a time course of 24, 48, 72 and 96 hours post-infection. The expression of *h*E-LecEGF was detected by sandwich ELISA with the monoclonal functional blocking antibody 7A9 and Western-blotting with the anti-flag antibody M2 as described in section 2.2.4.3. In Figure 3.18, *h*E-LecEGF was strongly expressed in Hi5 cells after transfection of 48 hours and then degraded. Under the same expression condition, High Five™ cells showed better expression of *h*E-LecEGF than Sf9 cells. This result is in agreement with that High Five™ cells are suitable for secretion of recombinant proteins[262].

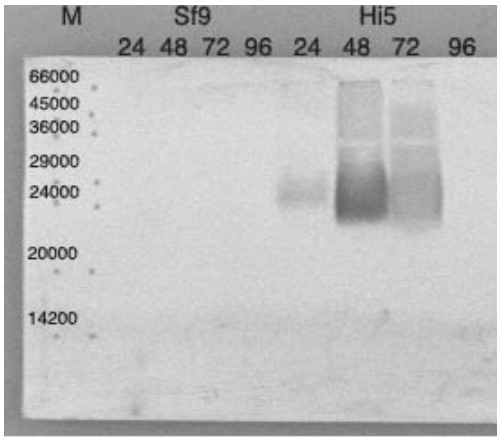


Figure 3.18. Western blotting analysis of the time course (post-infection of 24, 48, 72 and 96 hours) of expression of *h*E-LecEGF in Sf9 and Hi5 insect cells, detected with the monoclonal anti-flag M2 antibody. Lane M: Low molecular weight marker.

The best expression conditions of *h*E-LecEGF in Sf9 and Hi5 insect cells are shown in table 3.1.

Table 3.1. Optimized expression conditions of human E-LecEGF in Sf9 and Hi5 insect cells.

	Sf9	**High Five™**
Medium	Sf-900II SFM	Express Five SFM
Cell density (cells/ml)	$2x10^6$	$1x10^6$
MOI (1,2,5,10)	10	5
Post-infection time (hours)	72	48

3.2.5 Purification and characterization of *h*E-LecEGF expressed in insect cells

3.2.5.1 Monoclonal anti-E-selectin antibody 7A9 production, purification and coupling to sepharose 4B matrix

The monoclonal anti-E-selectin antibody 7A9 (mAb 7A9) is a functional blocking anti-E-selectin antibody[295,296]. In order to functionally purify the *h*E-LecEGF protein, mAb 7A9 was produced, purified, characterized and coupled to the sepharose as described below.

(1) Production of mAb 7A9

Hybridoma cell line 7A9 (ATCC Deposit No. HB-10135) expresses the monoclonal functional blocking anti-E-selectin antibody 7A9 (mAb 7A9). The cell line was recovered from liquid nitrogen and cultured in the RPMI-15 medium containing 15% FBS (recommended medium by the manufacturer).

In order to reduce the contamination of IgG from fetal bovine serum (FBS) and facilitate purification, the cells were adapted to grow in the medium RPMI-2 with 2% FBS (see section of 2.2.5.1). In addition, the special FBS serum containing ultra low IgG (5 μg/ml) was supplemented in the culture medium.

Monoclonal antibody 7A9 was produced in the RPMI-2 medium containing 2% FBS in both T175 Flasks and a roller bottle (1700 cm^2 expanded surface, Corning). In order to increase the yield of antibody, the culture supernatant was inoculated twice with cells as described in section 2.2.5.1.

(2) Purification of mAb 7A9

The produced mAb 7A9 in culture supernatant is an IgG1 subclass antibody[295,296]. Since the murine IgG1 antibody showed stronger binding affinity to Protein G matrix than Protein A, mAb 7A9 was purified by a Protein G column (Amersham Pharmacia) attached to an FPLC system as described in section 2.2.5.1. The purified mAb 7A9 was further characterized by SDS-PAGE, Bradford Assay, Western-blotting and ELISA methods.

(3) Characterization of mAb 7A9

- **SDS-PAGE analysis**

The purified mAb 7A9 was analyzed by 12% SDS-PAGE and silver staining (Figure 3.19). Under reducing conditions, two bands were shown on the gel, the upper band is the 55 kDa of the heavy chain of mAb 7A9 and the lower band is 25 kDa of the light chain. No contaminants were visible on the gel, indicating that highly pure mAb 7A9 was obtained.

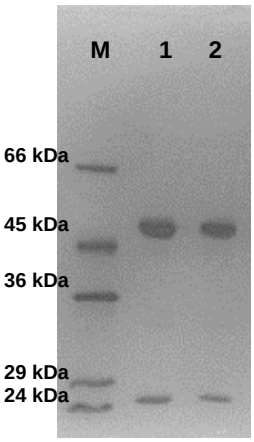


Figure 3.19. 12% SDS-PAGE analysis of the purified mAb 7A9 fraction 1 (Lane 1) and fraction 2 (Lane 2) under reducing condition and silver staining. Lane M: Low molecular weight marker.

- **Verification of mAb 7A9**

Three western-blotting analyses were performed to confirm the functionality of mAb 7A9 as described in section 2.2.5.1.

mAb 7A9 is a murine antibody

Reduced mAb 7A9 (1 μg) was blotted to the nitrocellulose membrane after separation by 12% SDS-PAGE. The recognition of mAb 7A9 by the anti-mouse IgG (whole molecule)–alkaline phosphatase antibody indicated that mAb 7A9 is a murine antibody (see Figure 3.20).

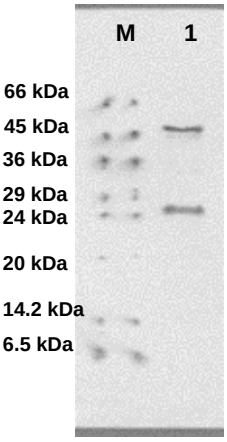


Figure 3.20. Western-blotting analysis of reduced mAb 7A9 with AP-Mouse IgG (whole molecule). Lane M: Low molecular weight marker.

mAb 7A9 is a functional blocking anti-E-selectin antibody

Western-blotting analysis of native and denatured *h*E-LecEGF (15 µg) with mAb 7A9 (10 µg/ml) were performed. The blotted *h*E-LecEGF on nitrocellulose membrane was verified by staining with Ponceau S solution prior to antibody detection. The recognition of native *h*E-LecEGF (Figure 3.21) and non-recognition of the denatured and reduced *h*E-LecEGF (Figure 3.22) by mAb 7A9 indicated that mAb 7A9 only recognizes the functional *h*E-LecEGF. This is in agreement with the previous report[296]. Unlike the others antibodies, a higher concentration of mAb 7A9 (10 µg/ml) was needed for identification of *h*E-LecEGF as reported by Ishii [334]. This could be due to the low avidity of mAb 7A9.

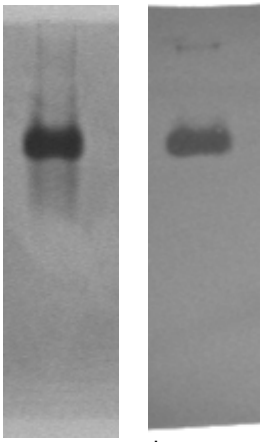


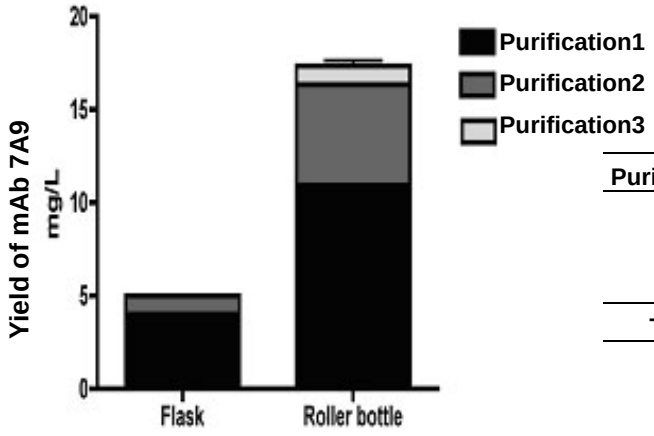
Figure 3.21. a. 8% Native-PAGE of the *h*E-LecEGF; b. Western-blotting result of the native *h*E-LecEGF, identified by mAb 7A9.

Figure 3.22. Western-blotting analysis of reduced and denatured *h*E-LecEGF identified by mAb 7A9. Lane M: Low molecular weight marker.

- **Yield determination**

The amount of purified mAb 7A9, estimated by Bradford micro-assay, was 17.3 mg from 1L culture supernatant in the roller bottle and 5 mg in the tissue flask with the same cell density of $5x10^5$ cells/ml. It showed that production in a roller bottle was three times higher than in tissue

flasks (Figure 3.23). Compared with the culture in T-flasks, the slow rolling of the roller bottle offers more CO_2 and nutrients in the culture, contributing to the higher production. The yield of 10.9 mg/L in the first-time purification of the supernatant, and of 5.4 mg/L in the second-time purification of the same supernatant showed that multiple purifications of the same supernatant were crucial for high yields of antibody (see Figure 3.24). This is in agreement with the previous report[323]. Although the binding capacity of the Protein G column is ~ 25 mg/ml of human IgG[335], only 10.9 mg/ml of mAb 7A9 was obtained from the first-time purification, it could be due to the lower binding capacity of the column to mouse IgG1 than human IgG1, or other IgGs from serum compete the binding sites on the column.



Purification	T-Flask (mg/L)	Roller bottle (mg/L)
1	4.1	10.9
2	0.9	5.4
3		1
Total	5	17.3

Figure 3.23. Yields of mAb 7A9 purified by the Protein G column, quantified by Bradford micro-assay.

(4) Coupling of mAb 7A9 to CNBr-activated Sepharose 4B matrix

Pure mAb 7A9 was coupled to cyanogen bromide activated sepharose 4B matrix for the functional purification of *h*E-LecEGF, according to the manufacturer's instructions.

Prior to the coupling, the purified mAb 7A9 was concentrated and buffer-exchanged to the coupling buffer (0.1M $NaHCO_3$, 0.5 M NaCl, pH 8.5) by Amicon Ultra-4 (10 kDa) centrifugal filter devices in order to remove the contaminants in the buffer, such as Tris and glycine (see section 2.2.5.1). 68% mAb 7A9 was recovered in the coupling buffer as shown in Table 3.2.

Table 3.2. Recovery of mAb 7A9 during the concentrating and buffer exchange.

mAb 7A9	Total protein (mg)	Recovery
Pure protein	15	
Concentration	12.8	85%
Buffer changing	10.2	68%

The coupling of mAb 7A9 to the matrix was performed as described in section 2.2.5.1. The coupling efficiency, determined by Bradford micro-assay, is ~ 97%. The mAb 7A9 coupled matrix was further analyzed on 12% SDS-PAGE under reducing conditions as described in section 2.2.5.1. On the gel, the antibody bands detected from the coupled matrix showed that sepharose 4B matrix was coupled with the mAb 7A9 (Figure 3.24).

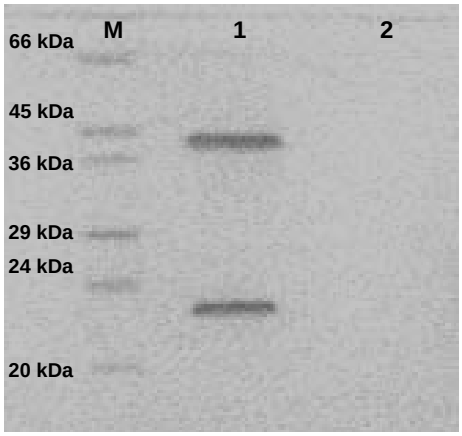


Figure 3.24. 12% SDS-PAGE analysis of mAb 7A9 coupled (Lane 1) and non-coupled CNBr activated sepharose 4B matrix (Lane 2) under reducing condition with commassie staining. Lane M: Low molecular weight marker.

In total, three mAb 7A9 coupled columns were prepared. 1 ml sepharose 4B matrix coupled with 7 mg mAb 7A9 was used for purification of *h*E-LecEGF expressed in insect cells; 2 ml and 5 ml matrix coupled with 14 mg and 20 mg mAb 7A9 respectively were used for *h*E-LecEGF expressed in CHO K1 cells.

3.2.5.2 Purification and characterization of *h*E-LecEGF by anti-E-selectin affinity chromatography

(1) Batch purification with mAb 7A9 coupled matrix

Functional purification of *h*E-LecEGF expressed in High Five™ and Sf9 insect cells was performed by batch purification with a 1 ml mAb 7A9 coupled sepharose matrix (see section 2.2.5.1) using the buffer described in Table 3.3. $CaCl_2$ (2 mM) was added in all purification buffers since the binding activity of *h*E-LecEGF is calcium-dependent.

Table 3.3. Buffers for the purification of *h*E-LecEGF with the mAb 7A9 coupled matrix.

	Composition
Binding buffer	50 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.5
Washing buffer	50 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100, 2 mM $CaCl_2$, pH 7.5
Elution buffer	100 mM Glycine, 2 mM $CaCl_2$, pH 3.5

(2) SDS-PAGE and Western-blotting analysis

*h*E-LecEGF purified by the mAb 7A9 coupled matrix was analyzed on 15% SDS-PAGE with silver staining. Under reducing conditions, two bands at molecular weight 24-29 kDa and one band at 66 kDa were shown on the gel (Figure 3.25). Western-blotting analysis was then performed to identify the bands on gel.

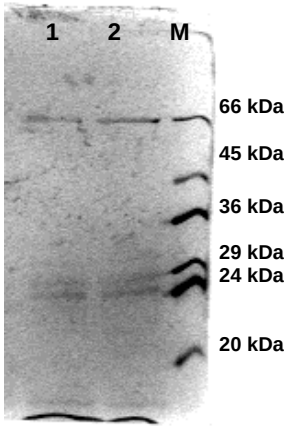


Figure 3.25. 15% SDS-PAGE analysis (reducing condition and silver staining) of the purified *h*E-LecEGF fraction 1 (Lane 1) and fraction 2 (Lane 2) by batch purification with the mAb 7A9 coupled sepharose 4B matrix. Lane M: Low Molecular weight marker.

Western-blotting analysis of the purified *h*E-LecEGF with monoclonal anti-flag M2 antibodies (SIGMA) is shown in Figure 3.26. The anti-flag M2 antibody was used for detection of the denaturing and reducing *h*E-LecEGF protein, since mAb 7A9 only recognizes the native *h*E-LecEGF. On the membrane, the main two bands recognized by the anti-flag antibody are at the molecular weight of 24-29 kDa (Figure 3.26), indicating the two bands with the same molecular weights on SDS-PAGE (Figure 3.25) represent the *h*E-LecEGF protein. The band at 66 kDa on the gel (Figure 3.25) could be contaminant BSA from serum. The two bands on the membrane could be the glycosylation isotype of *h*E-LecEGF, since it contains three potential N-glycosylation sites (Asn4, Asn124 and Asn139) as predicted in section 3.1.2.1. This result showed that the *h*E-LecEGF protein can be purified with the mAb 7A9 coupled matrix.

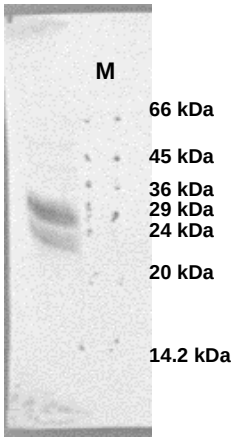


Figure 3.26. Western blotting analysis (with the monoclonal anti-flag M2 antibody) of *h*E-LecEGF purified by the mAb7A9 coupled matrix. Lane M: Low molecular weight marker.

3.2.5.3 Purification and characterization of *h*E-LecEGF by anti-flag affinity chromatography

(1) SDS-PAGE and Western-blotting analysis

*h*E-LecEGF expressed in insect cells was fused with an N-terminal flag tag (see section 3.2.1.2). Therefore, the purification of *h*E-LecEGF can be performed by affinity chromatography with the anti-flag antibody coupled matrix. A 2 ml anti-flag M2 monoclonal antibodies agarose (SIGMA) column was used for the purification of *h*E-LecEGF expressed in High Five™ and Sf9 cells by FPLC according to the manufacturer's instructions (see section 2.2.5.3). After washing with 10 bed-volumes of the washing buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.5), the eluted *h*E-LecEGF with the elution buffer (100 mM glycine, pH 3.5) was analyzed on 15% SDS-PAGE under reducing conditions and commassie staining (see Figure 3.27). The elution fractions (Lane 1 and 2) showed strong signals at the molecular weight of 24-29 kDa and several several bands of above 45 kDa on the gel (Figure 3.27).

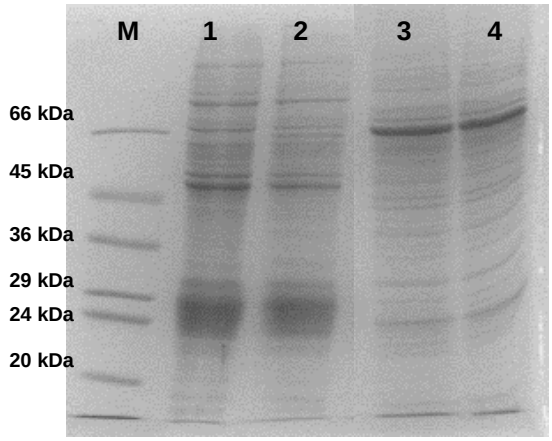


Figure 3.27. 15% SDS-PAGE analysis of *h*E-LecEGF purified by anti-flag affinity chromatography under reducing conditions and commassie staining. Lane M: Low molecular weight marker; Lane 1 and 2: The eluted fractions 1 and 2; Lane 3: The supernatant; Lane 4: The flow through.

Western-blotting analysis of the eluted *h*E-LecEGF was performed for the identification of *h*E-LecEGF (see section 2.2.5.3). On the membrane, the specific bands recognized by monoclonal anti-flag M2 antibodies (Sigma) are at the molecular weight of 24-29 kDa (Figure 3.28), indicating that the bands at the same molecular weights on the SDS-PAGE represent the recombinant protein of *h*E-LecEGF fused with the N-terminal Flag tag and there exists contaminants in the eluted fractions (Figure 3.27). Therefore, the anti-flag chromatography needs to be further optimized or other chromatographies should be performed in order to obtain the pure protein.

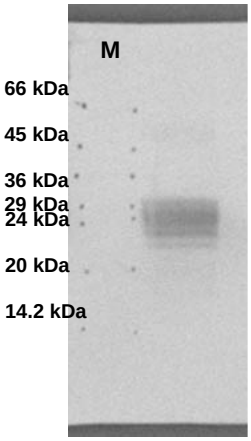


Figure 3.28. Western blotting analysis of *h*E-LecEGF purified by anti-flag chromatography with monoclonal anti-flag M2 antibodies. Lane M: Low molecular weight marker.

In Figure 3.26, the main two bands of *h*E-LecEGF purified by the mAb 7A9 coupled matrix were recognized by anti-flag antibodies, whereas four bands of *h*E-LecEGF purified by the anti-flag column were recognized by the same antibody (Figure 3.28). It indicated that *h*E-LecEGF purified by the anti-flag column might contain the non-correctly folded proteins, which were recognized by anti-flag antibodies, but not the functional blocking anti-E-selectin antibody mAb 7A9.

The molecular weight of *h*E-LecEGF fused with the N-terminal flag tag was predicted as 19.0792 kDa by the program ProtParam (see section 2.1.3). The purified *h*E-LecEGF showed four bands at the molecular weight of 24-29 kDa (Figure 3.28), indicating that *h*E-LecEGF expressed in insect cells was post-translationally modified.

In order to obtain pure protein in one-step purification by anti-flag affinity chromatography, the purification conditions of *h*E-LecEGF were further optimized as described in section 2.2.5.3. The purified *h*E-LecEGF was analyzed on 15% SDS-PAGE under reducing conditions and silver staining. Under the optimized condition (Table 3.4), pure protein *h*E-LecEGF was achieved by addition of 0.5% Triton X-100 to the washing buffer (see Figure 3.29). The non-ionic detergent Triton X-100 was used to reduce the non-specific binding of contaminating proteins which hydrophobically or ionically interact with the column.

Table 3.4. The optimized purification condition of *h*E-LecEGF by anti-flag affinity chromatography.

	Composition	Flow rate
Binding buffer	50 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.5	1.0 ml/min
Washing buffer	50 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100, 2 mM $CaCl_2$, pH 7.5	1.0 ml/min
Elution buffer	100 mM Glycine, 2 mM $CaCl_2$, pH 3.5	0.5-1 ml/min

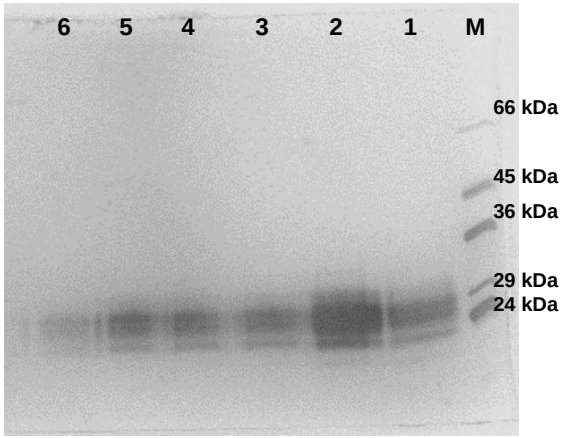


Figure 3.29. *h*E-LecEGF purified by the optimized anti-flag affinity chromatography on 15% SDS-PAGE and silver staining. Lane M: Low molecular weight marker; Lane 1-6: Eluted *h*E-LecEGF fractions 1-6.

(2) Monomeric identification

*h*E-LecEGF purified by the optimized anti-Flag affinity chromatography was analyzed on 15% SDS-PAGE under both reducing and non-reducing conditions and silver staining (Figure 3.30). According to the western-blotting result (Figure 3.28), the purified *h*E-LecEGF is pure. Oligomerization of *h*E-LecEGF under non-reducing conditions was not observed, indicating that the *h*E-LecEGF protein is a monomer. This result is in agreement with the previous report[336].

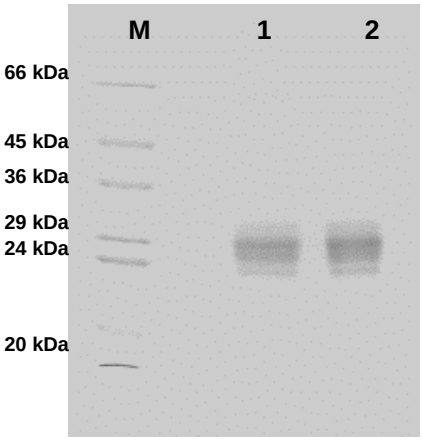
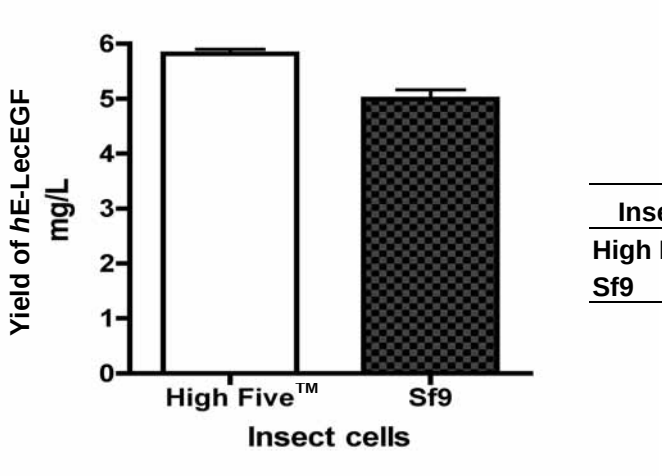


Figure 3.30. 15% SDS-PAGE analysis of the purified *h*E-LecEGF under non-reducing (Lane 1) and reducing (Lane 2) conditions and commassie staining. Lane M: Low molecular weight marker.

(3) Yield determination

The amount of the purified *h*E-LecEGF was quantified by Bradford micro-assay (see section 2.2.5.3). 5.82 mg/L of pure *h*E-LecEGF was obtained in High Five™ (Hi5) cells expression and 4.99 mg/L in Sf9 cells (Figure 3.31). Hi5 cells showed better production of *h*E-LecEGF than Sf9, confirming that High Five™ cells are suitable for secretion of proteins than other insect cells[262].



Insect cells	Medium	Yield (mg/L)
High Five™	Express Five SFM	5.82
Sf9	Sf-900II SFM	4.99

Figure 3.31. Yields of the purified *h*E-LecEGF from the expression in High Five™ and Sf9 cells.

(4) Activity determination

Western-blotting and HRP-ELISA were performed to identify the activities of the purified *h*E-LecEGF (see section 2.2.5.3). The native *h*E-LecEGF expressed in Hi-5 cells was recognized by the functional blocking anti-E-selectin mAb 7A9 in western-blotting (Figure 3.32) and in HRP-ELISA (Figure 3.33). Under the native condition, *h*E-LecEGF was separated not only by the size of protein, but also by the shape and charge, hence, it showed several bands on the membrane (Figure 3.32). The recognition of *h*E-LecEGF by the mAb 7A9 in both western-blotting and ELISA indicated that the purified *h*E-LecEGF is active.

Figure 3.32. Western-blotting analysis of the native *h*E-LecEGF expressed in Hi-5 cells with the functional blocking mAb 7A9.

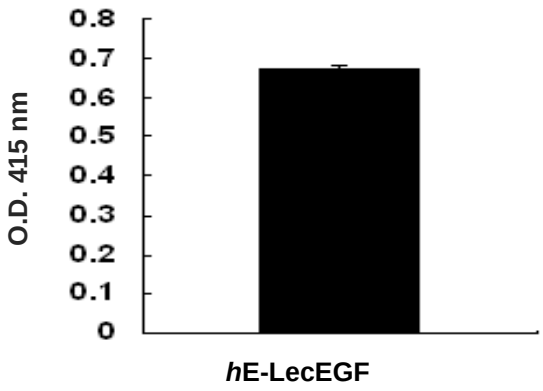


Figure 3.33. HRP ELISA analysis of the *h*E-LecEGF expressed in Hi-5 with the functional blocking mAb 7A9.

(5) Deglycosylation of *h*E-LecEGF

*h*E-LecEGF contains three potential N-glycosylation sites at Asn4, Asn124 and Asn139 as predicted by the NetNglyc1.0 server (see section 3.1.2.1). The purified *h*E-LecEGF showed four bands on SDS-PAGE at the molecular weight of 24-29 kDa rather than the predicted 19.07 kDa (Figure 3.29), indicating the post-translational glycosylation of the protein. In order to obtain the homogeneous *h*E-LecEGF, deglycosylation was performed.

- **Deglycosylation with PNGase F and N-Glycosidase A**

Peptide-N-glycosydase F (PNGase F, Roche) and N-glycosydase A (Roche) were used to remove the glycans of *h*E-LecEGF expressed in High five™ and Sf9 cells by either separately or combined. PNGase F is a widely used deglycosidase[281]. It recognizes the high-mannose, hybrid or complex type of glycans[281]. N-Glycosidase A recognizes all types of asparagine bound N-glycans[281] and the core alpha (1,3)-fucose modification in High Five™ cells, which was resistant to PNGase F.

Deglycosylations were performed at 37°C under native and denaturing conditions, respectively (see section 2.2.5.3). The deglycosylated *h*E-LecEGF was analyzed on 15% SDS-PAGE under reducing conditions and commassie staining. Under native and denaturing conditions, compared with the untreated *h*E-LecEGF protein, no apparent shift of *h*E-LecEGF was observed on gel after 96 hours' digestion with PNGase F (Figure 3.34) and N-Glycosidase A (Figure 3.35). The same pattern was also observed under native conditions by combination digestion of *h*E-LecEGF with these two deglycosidases (Figure 3.36).

Under native and denaturing conditions, the uniform deglycosylated *h*E-LecEGF was not obtained by deglycosylation with PNGase F and N-Glycosidase A. This result is similar to the previous report[324]. The *h*E-LecEGF protein expressed in insect cells showed resistance to these deglycosidases. The features of these deglycosidases may partly explain this result. PNGase F is sensitive to the protein conformation, especially when the asparagine residue sequon is on the amino or carboxyl end of a peptide[278,279,285]. N-Glycosidase A is not able to efficiently remove all susceptible oligosaccharides on proteins, e.g. ovalbumin and orosomucoid. This could be due to the large size of the enzyme (66.8 kDa), which may impair its access to cleavage sites, or to the fact that the N-glycosyl residue is located on the carboxyl or amino terminal end of a peptide[285]. The limitation of these enzymes might influence the removal of glycans from *h*E-LecEGF.

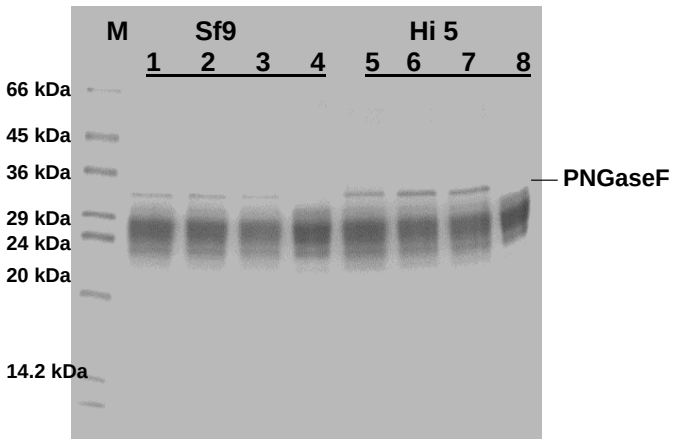


Figure 3.34. 15% SDS-PAGE analysis (reducing condition and commassie staining) of deglycosylation of the *h*E-LecEGF expressed in Sf9 and Hi5, treated with PNGase F for 96 h under native and denaturing conditions. Lane M: Low molecular weight marker; Lane 1-3: Deglycosylated *h*E-LecEGF expressed in Sf9, Lane 1: Native conditions. Lane 2,3: Denaturing conditions, Lane 4: Untreated *h*E-LecEGF expressed in Sf9; Lane 5-7: Deglycosylated *h*E-LecEGF expressed in Hi5, Lane 5: Native conditions. Lane 6,7: Denaturing conditions, Lane 8: Untreated *h*E-LecEGF expressed in Hi5.

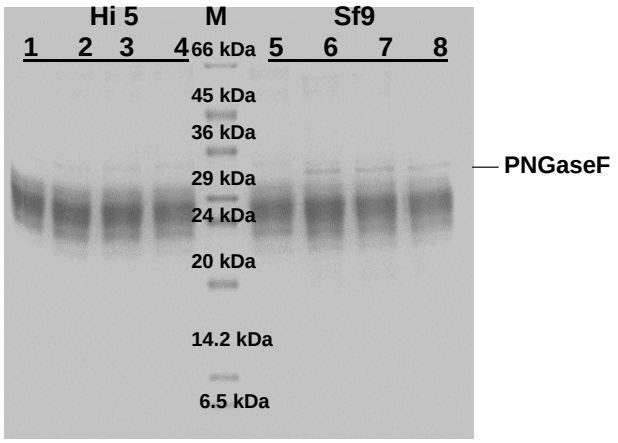


Figure 3.35. 15% SDS-PAGE analysis (reducing condition and commassie staining) of deglycosylation of the *h*E-LecEGF expressed in Sf9 and Hi5, treated with N-glycosidase A for 96 h under native and denaturing condition. Lane M: Low molecular weight marker; Lane 1: Untreated *h*E-LecEGF expressed in Hi5; Lane 2-4: Deglycosylated *h*E-LecEGF expressed in Hi5, Lane 2: Native conditions, Lane3,4: Denaturing conditions, Lane 5: Untreated *h*E-LecEGF expressed in Sf9; Lane 6-8: Deglycosylated *h*E-LecEGF expressed in Sf9, Lane 6: Native conditions, Lane 7,8: Denaturing conditions.

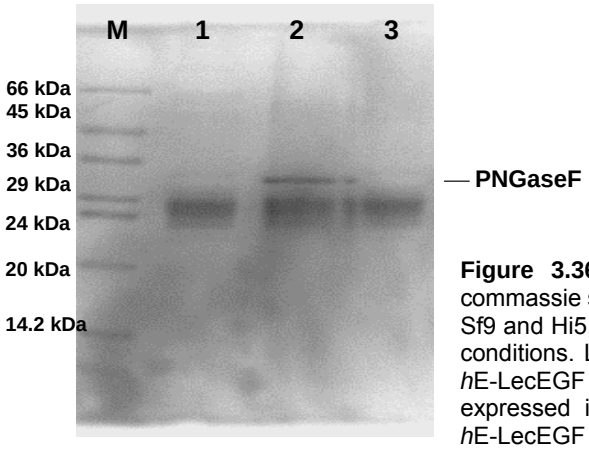


Figure 3.36. 15% SDS-PAGE analysis (reducing conditions and commassie staining) of deglycosylation of the *h*E-LecEGF expressed in Sf9 and Hi5, treated with N-glycosidase A and PNGase F under native conditions. Lane M: Low molecular weight marker; Lane 1: Untreated *h*E-LecEGF expressed in Hi5; Lane 2: Deglycosylated *h*E-LecEGF expressed in Sf9 under native conditions; Lane 3: Deglycosylated *h*E-LecEGF expressed in Hi5 under native conditions.

- **Deglycosylation with O-glycosidase**

*h*E-LecEGF expressed in Hi5 and Sf9 cells was also treated with O-glycosidase under native conditions (see section 2.2.5.3). The deglycosylated *h*E-LecEGF was analyzed on 15% SDS-PAGE under reducing conditions with Commassie staining. No obvious shift was found between the *h*E-LecEGF and the deglycosylated *h*E-LecEGF (Figure 3.37), indicating that no O-linked glycan was removed, coinciding with the prediction that there is no O-glycosylation site in the *h*E-LecEGF (see section 2.1.2.2).

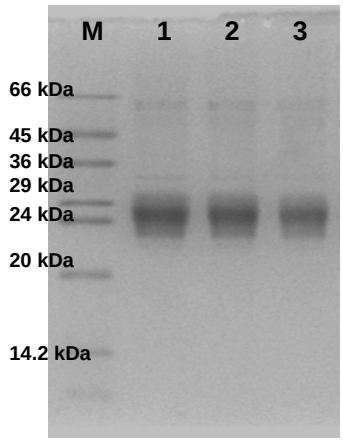


Figure 3.37. 15% SDS-PAGE analysis (reducing conditions and commassie staining) of the deglycosylated *h*E-LecEGF expressed in Hi5 (Lane 1) and Sf9 cells (Lane 2), treated with O-glycosidase under native condition.Lane M: Low molecular weight marker; Lane 3: Untreated *h*E-LecEGF expressed in Hi5.

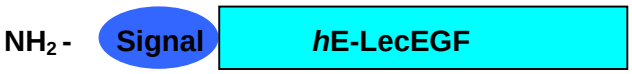
(6) Glycans detection

Detection of glycans of *h*E-LecEGF and *h*E-LecEGF treated with deglycosidases was performed with a GelCode Glycoprotein Staining Kit (Pierce) as described in section 2.2.5.3. The magenta bands of the deglycosylated *h*E-LecEGF on gel indicated that glycans of *h*E-LecEGF expressed in either Hi5 or Sf9 cells were not removed by PNGase F and N-glycosidase A under native or denaturing conditions (data not shown, due to the problem of documentation). This result might be due to the restriction of the deglycosidases as discussed above.

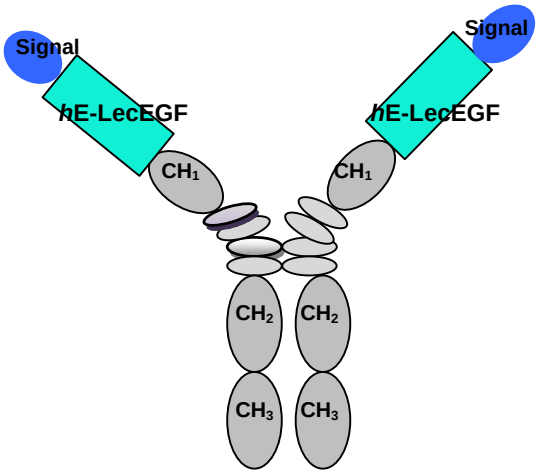
3.3 Cloning, Expression, Purification and Characterization of human E-LecEGF in CHO-K1 cells

3.3.1 Cloning of *h*E-LecEGF in CHO K1 cells

*h*E-LecEGF was then expressed in CHO K1 cells, since the uniform *h*E-LecEGF was not obtained in the insect cells expression system (see section 3.2). Two *h*E-LecEGF expression clones were generated. One clone expresses *h*E-LecEGF without tag and can be functionally purified by the mAb 7A9 coupled column (Figure 3.38a). The other contains the partial CH_1 domain and complete CH_2 and CH_3 domains of human IgG1 (*h*IgG1 tag) like the previously reported E-selectin/IgG chimeras (the extracellular domains of human E-selectin fused with a C-terminal *h*IgG1 tag)[333], which can be purified by a Protein A column (Figure 3.38b).



a.



b.

Figure 3.38. Schematic representation of *h*E-LecEGF constructs expressed in CHO K1cell, the signal will be deleted when protein is secreted to the medium. a: the *h*E-LecEGF expressed without tag; b: the *h*E-LecEGF/IgG expressed with a *h*IgG1 tag.

3.3.1.1 Construction of the expression plasmid pYJE

The expression plasmid pYJE was constructed for expression of *h*E-LecEGF without tag.

(1) Cloning of *h*E-LecEGF into the vector pcDNA3.1 (+)

In order to secret the *h*E-LecEGF protein into the medium, the signal, lectin and EGF-like domains of human E-selectin (SLE sequence, 534bp) were amplified by PCR from the genomic DNA of CHO-E-sel/IgG cells with the forward primer E1 containing a *Bam*H I restriction endonuclease site and the backward primer E2 containing a stop codon downstream of the SLE sequence and an *EcoR* I site as described in section 2.3.2.2. The PCR product was analyzed on 2% agarose gel (Figure 3.39, Lane E1). Using the restriction/ligation cloning method, the PCR fragment of the SLE sequence was inserted into the vector pcDNA3.1 (+) (5428bp) to construct the expression plasmid pYJE (5899bp, Figure 3.40). The expression of *h*E-LecEGF was under the control of the CMV promoter (P_{CMV}) as shown in Figure 3.40.

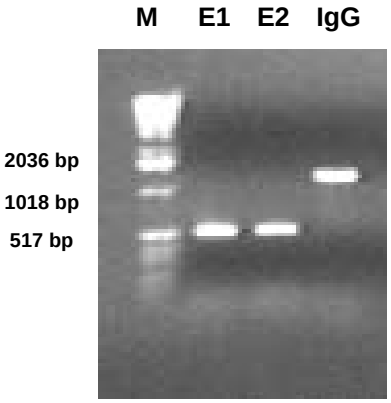


Figure 3.39. PCR products of *h*E-LecEGF and *h*IgG1 tag with *pfu* DNA polymerase on 2% agarose gel stained with ethidium bromide. Lane M: DNA marker X; Lane E1: Amplified *h*E-LecEGF fragment with E1and E2 primers; Lane E2: Amplified *h*E-LecEGF fragment with E1and E3 primers; Lane IgG: Amplified human IgG1 tag with IgG1 and IgG2 primers.

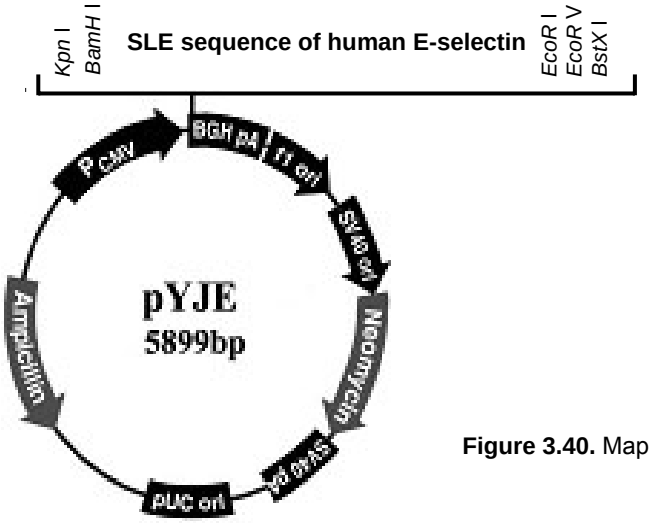


Figure 3.40. Map of the plasmid pYJE.

(2) Characterization of the plasmid pYJE

The pYJE constructs were confirmed by colony PCR and DNA sequencing methods (see section 2.3.2.6).

- **Colony PCR**

The SLE sequence of human E-selectin was amplified by colony PCR from recombinant clones (C1-C3) with E1 and E2 primers and *Taq* DNA polymerase. The amplified PCR products (534 bp) indicated the presence of the SLE sequence in the recombinant clones (see Figure 3.41).

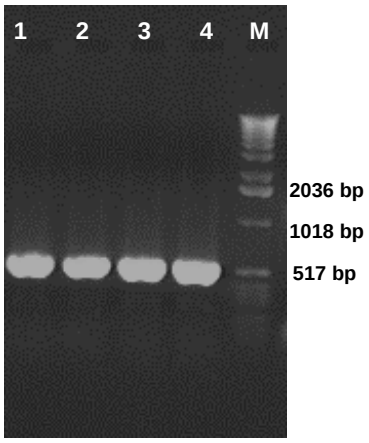


Figure 3.41. The SLE fragments amplified by Colony PCR on 1.5% agarose gel with E1 and E2 primers and *Taq* DNA polymerase. Lane M: DNA marker X; Lane 1: Positive control, amplified from the genomic DNA of CHO-E-sel-IgG cells; Lane 2-4: Recombinant clones C1-3.

- **DNA sequencing of the plasmid pYJE**

Two constructs of pYJE were sequenced with T7 primer for plus strand determination as described in section 2.3.2.6. 100% identity of the SLE sequence (534 bp) in the alignment result indicated that both two plasmids pYJE were correct (Figure 3.42).

```
Query  44   GGATCCACCATGATTGCTTCACAGTTTCTCTCAGCTCTCACTTTGGTGCTTCTCATTAAA  103
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GGATCCACCATGATTGCTTCACAGTTTCTCTCAGCTCTCACTTTGGTGCTTCTCATTAAA  60

Query  104  GAGAGTGGAGCCTGGTCTTACAACACCTCCACGGAAGCTATGACTTATGATGAGGCCAGT  163
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GAGAGTGGAGCCTGGTCTTACAACACCTCCACGGAAGCTATGACTTATGATGAGGCCAGT  120

Query  164  GCTTATTGTCAGCAAAGGTACACACACCTGGTTGCAATTCAAAACAAAGAAGAGATTGAG  223
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GCTTATTGTCAGCAAAGGTACACACACCTGGTTGCAATTCAAAACAAAGAAGAGATTGAG  180

Query  224  TACCTAAACTCCATATTGAGCTATTCACCAAGTTATTACTGGATTGGAATCAGAAAAGTC  283
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  TACCTAAACTCCATATTGAGCTATTCACCAAGTTATTACTGGATTGGAATCAGAAAAGTC  240

Query  284  AACAATGTGTGGGTCTGGGTAGGAACCCAGAAACCTCTGACAGAAGAAGCCAAGAACTGG  343
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  AACAATGTGTGGGTCTGGGTAGGAACCCAGAAACCTCTGACAGAAGAAGCCAAGAACTGG  300

Query  344  GCTCCAGGTGAACCCAACAATAGGCAAAAAGATGAGGACTGCGTGGAGATCTACATCAAG  403
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  GCTCCAGGTGAACCCAACAATAGGCAAAAAGATGAGGACTGCGTGGAGATCTACATCAAG  360

Query  404  AGAGAAAAAGATGTGGGCATGTGGAATGATGAGAGGTGCAGCAAGAAGAAGCTTGCCCTA  463
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  AGAGAAAAAGATGTGGGCATGTGGAATGATGAGAGGTGCAGCAAGAAGAAGCTTGCCCTA  420

Query  464  TGCTACACAGCTGCCTGTACCAATACATCCTGCAGTGGCCACGGTGAATGTGTAGAGACC  523
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Sbjct  421  TGCTACACAGCTGCCTGTACCAATACATCCTGCAGTGGCCACGGTGAATGTGTAGAGACC  480

Query  524  ATCAATAATTACACTTGCAAGTGTGACCCTGGCTTCAGTGGACTCAAGTGTGAGCAAATT  583
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  ATCAATAATTACACTTGCAAGTGTGACCCTGGCTTCAGTGGACTCAAGTGTGAGCAAATT  540

Query  584  GTGTAAGAATTC  595
            ||||||||||||
Sbjct  541  GTGTAAGAATTC  552
```

Figure 3.42. Bl2seq alignment of the plus strand sequence of the pYJS (Query) and the SLE sequence of human E-selectin extracted from GenBank (sbjct). The identity was 100% in the alignment. The signal sequence is shown in grey italics and the hE-LecEGF in black bold.

3.3.1.2 Construction of the expression plasmid pYJEG

(1) Construction of the plasmid pYJ-IgG

In order to purify *h*E-LecEGF with a Protein A column, the plasmid pYJ-IgG was first constructed to carry the *h*IgG1 tag.

The *h*IgG1 tag (1020 bp) was amplified by PCR from the genomic DNA of CHO-E-sel/IgG cells with 5' primer IgG1 containing an *Eco*R I restriction endonuclease site and 3′ primer

IgG2 containing a stop codon downstream of the sequence of the *h*IgG1 tag and an *Xho* I site. The amplified fragments of the *h*IgG1 tag (Figure 3.39, Lane IgG) were then cloned into the plasmid pcDNA3.1(+) by a restriction/ligation cloning method to get the plasmid pYJ-IgG (Figure 3.43).

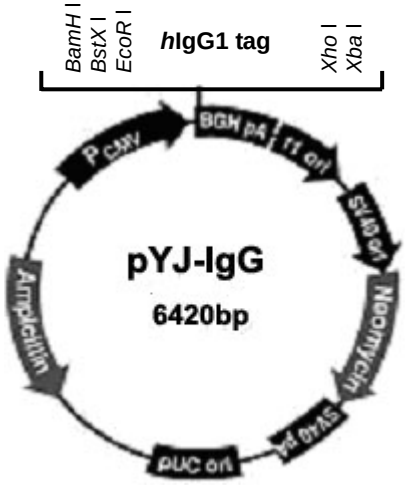


Figure 3.43. Map of the plasmid pYJ-IgG.

The purified constructs of pYJ-IgG were further confirmed by colony PCR and sequence verification as described in section 2.3.3.1. The amplified fragments of the *h*IgG1 tag from pYJ-IgG1-3 clones in colony PCR (Figure 3.44) and DNA sequencing results showed that pYJ-IgG constructs contained the human IgG1 tag.

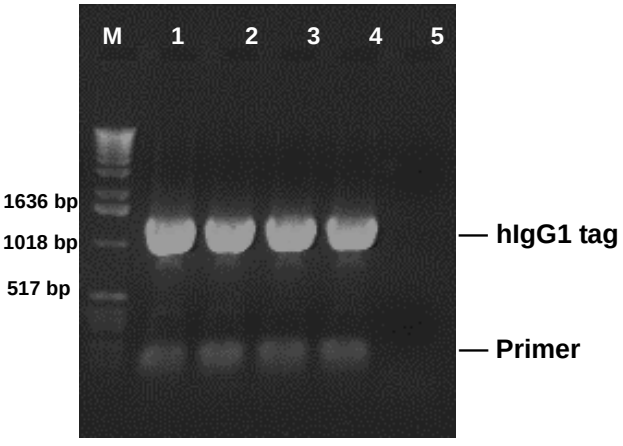


Figure 3.44. Colony PCR amplified fragments of the *h*IgG1 tag on 1.2% agarose gel with IgG1 and IgG2 primers and *Taq* DNA polymerase. Lane M: DNA marker X; Lane 1: Positive control, amplified from the genomic DNA of CHO-E-sel-IgG cells; Lane 2-4: Clones of pYJ-IgG1-3; Lane 5: Negative control.

(2) Cloning of the SLE sequence into the vector pYJ-IgG

The expression plasmid pYJEG was constructed to carry the SLE sequence of human E-selectin fused with a C-terminal *h*IgG1 tag. An enterokinase cleavage site was introduced between the *h*E-LecEGF encoding gene and the *h*IgG1 tag for further complete removal of the tag.

The forward primer E1 containing a *Bam*H I restriction endonuclease site and the backward primer E3 containing a protease enterokinase site (DDDDK) and an *Eco*R I site were used to amplify the SLE sequence from the genomic DNA of CHO-E-sel/IgG cells. The PCR products (Figure 3.39, Lane E2) were then cloned into the vector of pYJ-IgG (see section 2.3.3.2) to get the expression plasmids pYJEG (Figure 3.45). The constructs of pYJEG were further confirmed by colony PCR and sequencing verification.

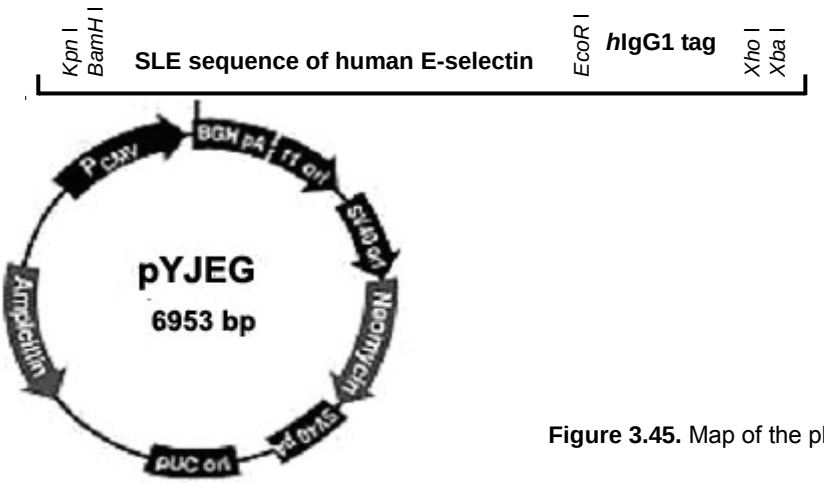


Figure 3.45. Map of the plasmid pYJEG.

3.3.2 Transfection and selection of the high, stable expression clones and subclones of CHO-YJE and CHO-YJEG

3.3.2.1 Determination of geneticin sensitivity

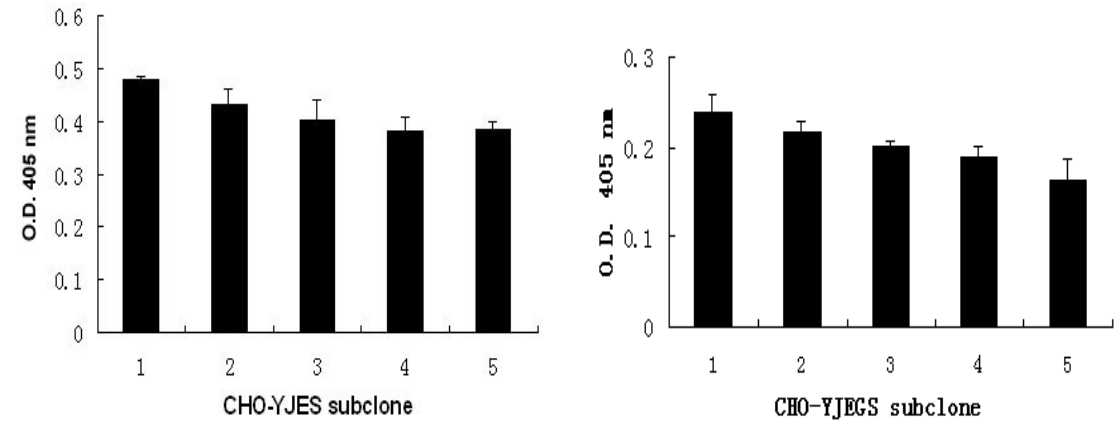
In order to select the stable expression clones and subclones, the sensitivity of CHO K1 cells to the antibiotic geneticin was determined by the method described in section 2.3.4.2. A concentration of 500 μg/ml of geneticin was chosen for the selection.

3.3.2.2 Transfection and selection

Prior to transfection, the expression plasmids pYJE and pYJEG were linearized by restriction endonuclearase *Sca* I, in order to decrease the likelihood of vector integration into the genome in such a way that disrupts the gene of interest or other elements required for the expression in mammalian cells. Restriction enzyme *Sca* I was chosen for linearization of the expression plasmids since this site is contained only in the ampicillin gene of the plasmids, not in the essential genes for expression (see section 2.3.4.3).

CHO K1 cells (viability >95%) were stably transfected with the linearized expression plasmids of pYJE or pYJE-IgG to generate the expression clones of CHO-YJE and CHO-YJEG, respectively. Lipid (metafectene)-mediated transfection was performed according to the manufacturer's protocol described in section 2.3.4.4. Selective medium containing 500 μg/ml geneticin was applied for selection of stable transfectants. High expression clones of CHO-YJE and CHO-YJEG were detected by AP-ELISA with mAb 7A9 post-transfection of two weeks (see section 2.3.5.1). AP-ELISA screenings were performed twice to avoid the false positive clone. Overall, for each construct, 10 high expression stable clones were obtained by screening 576 clones. After propagation, these generated clones were stored at –185°C in liquid nitrogen for long-term storage.

In order to reduce costs and facilitate purification, high-expression stable clones of CHO-YJE and CHO-YJEG were adapted to grow in DMEM:F12 selective medium containing 5% FBS instead of Ham's F12 selective medium containing 10% FBS, before screening of the subclones (see section 2.3.5.2). High expression subclones CHO-YJES and CHO-YJEGS from a single cell were selected by limiting dilution and detected twice by AP-ELISA (see section 2.3.5.2). Five high, stable expression subclones of CHO-YJES and CHO-YJEGS were achieved. The AP-ELISA results of these subclones are shown in Figure 3.45. CHO-YJES1 and CHO-YJEGS1 showed better expression than CHO-YJES 2-5 (Figure 3.46a) and CHO-YJEGS 2-5 (Figure 3.46b). Therefore, CHO-YJES1 and CHO-YJEGS1 were further characterized.



a. b.

Figure 3.46. The *h*E-LecEGF expression in CHO-YJES (a) and CHO-YJEGS (b) subclones determined by AP-ELISA.

3.3.2.3 Characterization of subclones CHO-YJES1 and CHO-YJEGS1

The best expression subclones CHO-YJES1 and CHO-YJEGS1 were characterized on both DNA level and protein level for the expression of *h*E-LecEGF and *h*E-LecEGF/IgG.

(1) Characterization on DNA level

The SLE sequence was amplified by colony PCR from the CHO-YJES1 subclone with E1 and E2 primers as described in section 2.3.5.3. The amplified fragments of the SLE sequence indicated that the SLE sequence was integrated into the genome of CHO-YJES1 subclone (Figure 3.47, Lane1).

Using the same method, the CHO-YJEGS1 subclone was confirmed from the presence of the SLE sequence and the *h*IgG1 tag by PCR amplification with E1 and E3 primers, and IgG1 and IgG2 primers (Figure 3.47, Lane 3 and 4).

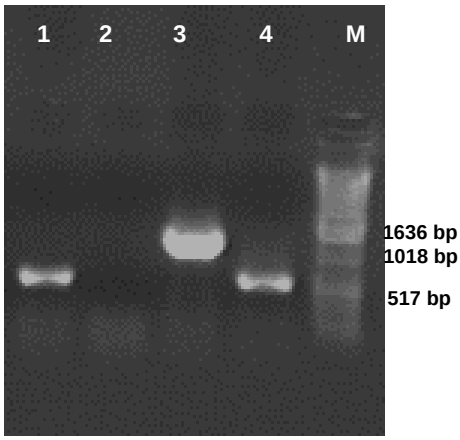


Figure 3.47. PCR products of the SLE sequence and the *h*IgG1 tag by colony PCR from the CHO-YJES1 and CHO-YJEGS1 subclones on 1.5% agarose gel. Lane M: DNA marker X; Lane 1: The SLE sequence amplified from CHO-YJES with E1 and E2 primers; Lane 2: Negative control (PCR amplified the SLE sequence from CHO-K1 cells with E1 and E2 primers); Lane 3: The *h*IgG1 tag amplified from the CHO-YJEGS subclone with IgG1 and IgG2 primers; Lane 4: The SLE sequence amplified from the CHO-YJES subclone with E1 and E3 primers.

(2) Characterization on protein level

- **Characterization of the CHO-YJES1 subclone**

100 ml culture supernatant of subclone CHO-YJES1 was collected and purified on a 2 ml mAb 7A9 coupled column (see section 2.2.5.1) attached to a FPLC system (see section 2.3.5.3). The eluted *h*E-LecEGF was further analyzed by the western-blotting method. Identical amounts of reduced and non-reduced *h*E-LecEGF (~1 μg) were separated by SDS-PAGE and blotted to the nitrocellulose membrane. 2 μg/ml of anti-E-selectin antibody N18 (Santa-Cruz Biotechnology) was used to detect the presence of *h*E-LecEGF (see section 2.3.5.3), since this antibody recognizes the denaturing and reducing *h*E-LecEGF protein. The result (Figure 3.48) showed that the bands at a molecular weight of ~ 23 kDa under reducing and non-reducing conditions were visible on the membrane, indicating that the CHO-YJES1 subclone expresses the monomeric *h*E-LecEGF protein.

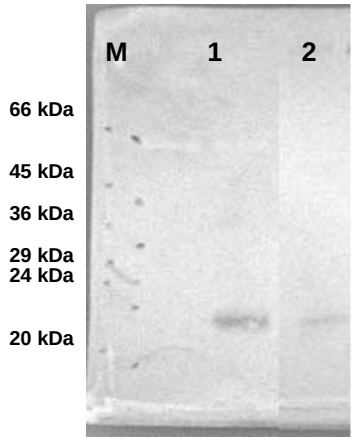


Figure 3.48. Western-blotting analysis of non-reduced (Lane 1) and reduced *h*E-LecEGF (Lane 2) purified from the expression of CHO-YJES1 subclone with the anti-E-selectin antibody N18. Lane M: Low Molecular weight marker.

- **Characterization of the CHO-YJEGS1 subclone**

Using the same characterization method of the CHO-YJES1 subclone, *h*E-LecEGF/IgG expressed from the CHO-YJEGS1 subclone was purified on a 1 ml Protein A column and characterized by SDS-PAGE and western-blotting analysis (see section 2.3.5.3).

12% SDS-PAGE analysis of the purified *h*E-LecEGF/IgG from 100 ml culture supernatant of subclone CHO-YJEGS is shown in Figure 3.49. Under reducing conditions, two bands at molecular weight ~ 66 kDa and one band at ~ 55 kDa are visible. The band at ~55 kDa could be a heavy chain of bovine IgGs derived from serum. Two bands at ~ 66 kDa could be *h*E-LecEGF/IgG or BSA contaminant. Under non-reducing conditions, four bands were detected. One band was at molecular weight ~155 kDa, which could be from bovine IgGs. Two bands at ~130 kDa and one band at ~66 kDa could be from *h*E-LecEGF/IgG. This result

indicated that additional gel filtration was needed to get the pure *h*E-LecEGF/IgG protein and remove the contaminants derived from serum. Western-blotting was performed to identify the *h*E-LecEGF/IgG protein on the gel.

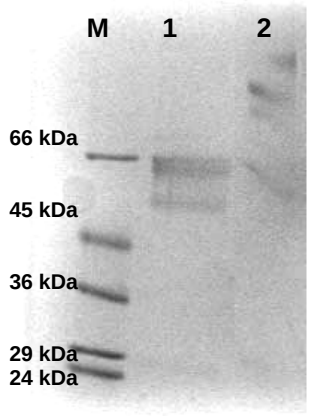


Figure 3.49. 12% SDS-PAGE analysis of *h*E-LecEGF-IgG purified from the CHO-YJEGS1 culture supernatant under reducing (Lane 1) and non-reducing (Lane 2) conditions with commassie staining. Lane M: Low Molecular weight marker.

In Western-blotting analysis, the recognition of reduced and non-reduced *h*E-LecEGF/IgG (~1 μg) by anti-human IgG monoclonal antibodies (1 μg/ml) is shown in Figure 3.50. The anti-human IgG monoclonal antibody was used to identify the *h*E-LecEGF/IgG protein since the protein fused with a C-terminal *h*IgG1 tag. Under reducing conditions, one band with molecular weight of ~ 66 kDa was detected on the membrane. Under non-reducing conditions, two main bands of ~130 kDa were visible due to the dimer of the protein formed under non-reducing conditions by the C-terminal *h*IgG1 tag fused to the protein. The non-uniform bands could be due to the glycosylation isotype of the protein. This result indicated that the subclone of CHO-YJEGS expresses the *h*E-LecEGF/IgG protein fused with a *h*IgG1 tag.

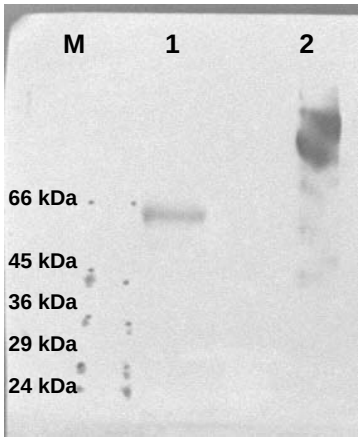


Figure 3.50. Western-blotting analysis of *h*E-LecEGF/IgG purified from the CHO-YJEGS1 subclone culture under reducing (Lane 1) and non-reducing (Lane 2) conditions with the anti-human IgG monoclonal antibody conjugated with alkaline phosphatase. Lane M: Low molecular weight marker.

3.3.3 Expression of *h*E-LecEGF from the CHO-YJES1 subclone

Compared with the *h*E-LecEGF/IgG protein expressed by the CHO-YJEGS subclone, *h*E-LecEGF expressed by CHO-YJES can be purified in one-step by the mAb 7A9 coupled column. Since it did not contain the tag (which could have the interference in structural studies), the best expression subclone CHO-YJES1 was used for further expression of *h*E-LecEGF (see section 3.3.2.2 and 3.3.2.3).

Sodium butyrate has been reported to increase the expression of proteins regulated by some mammalian promoters, including cytomegalovirus (CMV) and simian virus 40 (SV40) promoters[337,338]. However, the usefulness of sodium butyrate for improving production is limited since it rapidly induces cellular apoptosis[339]. Since expression of *h*E-LecEGF is under the control of CMV promoter, the effect of sodium butyrate on the production of *h*E-LecEGF was tested as described in section 2.3.6.3. Sodium butyrate (1 mM, 2 mM and 4 mM) was added respectively in the production phase in T-flask production. The expression of *h*E-LecEGF was evaluated by AP-ELISA (see section 2.3.5.1) and the cell viability was determined by Trypan blue method (see section 2.2.3.1). The net production of *h*E-LecEGF after addition of sodium butyrate at all concentrations was lower than the culture without supplement of sodium butyrate. The cell viability also reduced rapidly from 95% to 0% in two weeks at all concentrations of sodium butyrate. This could be due to the cytotoxic effect of sodium butyrate[339,340]. Hence, the production of *h*E-LecEGF was performed without addition of sodium butyrate.

The *h*E-LecEGF protein was produced in the DMEM:F12 selective medium containing 5% FCS in both T175cm^2-flasks and roller bottles (1700cm^2 expanded surface, corning) at 37°C and 5% CO_2 in a humid incubator as described in section 2.3.6.

3.3.4 Purification and characterization of *h*E-LecEGF expressed in CHO K1 cells

3.3.4.1 Purification and SDS-PAGE analysis

Column purification of *h*E-LecEGF expressed from the subclone CHO-YJES1 was performed with a 2 ml or 5 ml mAb 7A9 coupled column by FPLC as described in section 2.3.7.1. Purified *h*E-LecEGF was analyzed on 15% SDS-PAGE with silver staining (Figure 3.51). Only one band at molecular weight ~22 kDa was observed on the gel, indicating that pure *h*E-LecEGF can be obtained by one-step purification with the mAb 7A9 coupled column.

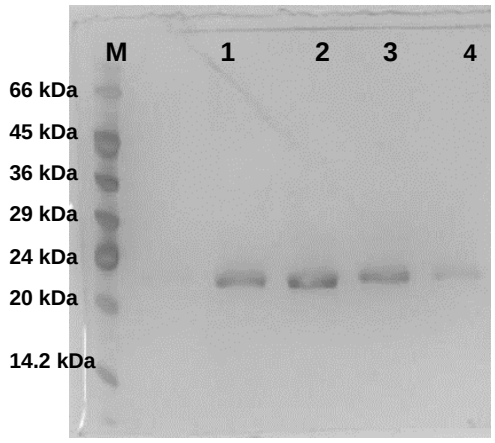


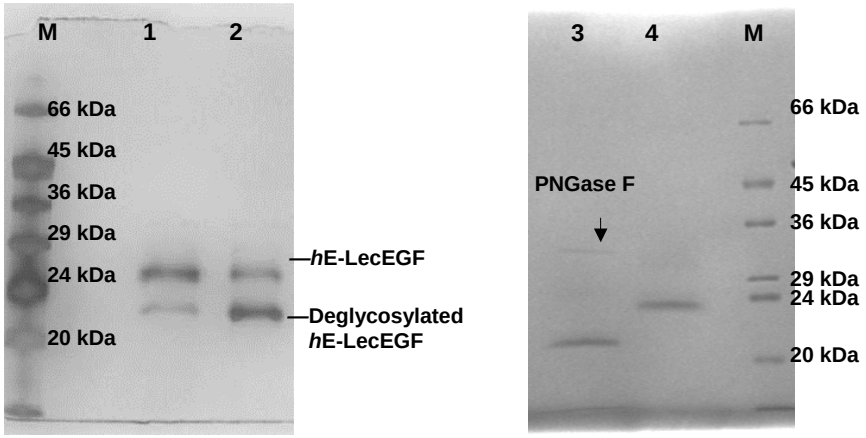
Figure 3.51. 15% SDS-PAGE analysis of *h*E-LecEGF purified by the mAb 7A9 coupled column under reducing conditions and silver staining. Lane M: Low molecular weight marker; Lane 1-4: Elution fractions 1-4.

3.3.4.2 Deglycosylation of *h*E-LecEGF expressed in CHO-K1 cells

*h*E-LecEGF contains three potential N-linked glycosylation sites (see section 3.1.2.1). The purified *h*E-LecEGF showed a higher molecular weight of ~ 22 kDa (Figure 3.51) than the predicted 18.1 kDa, indicating *h*E-LecEGF expressed in CHO K1 cells is post-translationally N-glycosylated. Therefore, deglycosylation of *h*E-LecEGF was performed in order to facilitate the future crystallization, since glycans on the protein could increase the solubility of protein in the buffer.

(1) Deglycosylation

Since the binding of *h*E-LecEGF to the ligands is calcium-dependent, deglycosylation of *h*E-LecEGF was performed under native conditions at 37 ºC with PNGase F (Roche) in a Tris buffer instead of the phosphate buffer (recommended by the manufacturer) to avoid the precipitation of calcium phosphate (see section 2.3.7.2). The time course of deglycosylation of *h*E-LecEGF was analyzed on post-digestion of 24, 48 and 72 hours by 15% SDS-PAGE gel and silver staining (Figure 3.52). Compared with the post digestion of 24 and 48 hours (Figure 3.52a), only the deglycosylated *h*E-LecEGF band was observed on gel after 72 hours digestion (Figure 3.52b). The decrease in the molecular weight of the deglycosylated *h*E-LecEGF implies the removal of glycans of *h*E-LecEGF expressed in CHO K1 cells. The negative result of the glycan detection with a GelCode Glycoprotein Staining Kit (Pierce) also indicated that no glycan was detected from the deglycosylated *h*E-LecEGF post-digestion of 72 hour (see section 2.3.7.2). All these results implied that glycans on *h*E-LecEGF (10 μg) were completely removed by 4U PNGase F in the Tris buffer after 72 hours digestion.



a. b.

Figure 3.52. 15% SDS-PAGE analysis of the time-course of post digestion of 24h (Lane 1), 48h (Lane 2) and 72h (Lane 3) of the deglycosylated *h*E-LecEGF under reducing conditions and silver staining. Lane 4: The untreated *h*E-LecEGF; Lane M: Low molecular weight marker.

(2) Purification of deglycosylated *h*E-LecEGF and SDS-PAGE analysis

The deglycosylated *h*E-LecEGF was further purified with a Sepharose Q (0.5 ml) column in order to remove the PNGase F (see section 2.3.7.2). The purified deglycosylated *h*E-LecEGF was analyzed on 15% SDS-PAGE under non-reducing conditions and silver staining (Figure 3.53). Pure deglycosylated *h*E-LecEGF was present in the flow through.

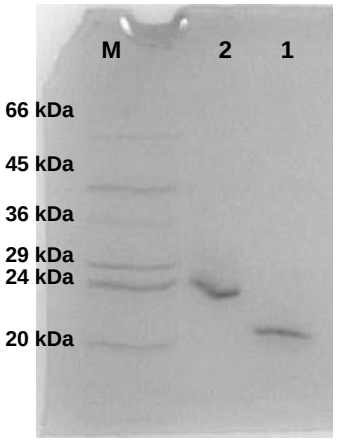


Figure 3.53. 15% SDS-PAGE analysis of the deglycosylated *h*E-LecEGF purified by 0.5 ml sepharose Q column under non-reducing condition (Lane 1); Lane 2: *h*E-LecEGF; Lane M: Low molecular weight marker.

3.3.4.3 Protein identification by western-blotting analysis

Reduced and non-reduced *h*E-LecEGF (1 μg) and deglycosylated *h*E-LecEGF (1 μg) were identified by western-blotting with the anti-E-selectin antibody N18 (2 μg/ml) as described in section 2.3.7.3 (Figure 3.54). On the nitrocellulose membrane, the reduced and non-reduced *h*E-LecEGF showed a single specific band at molecular weight ~ 22 kD. The reduced and non-reduced deglycosylated *h*E-LecEGF showed a single band at ~20 kDa. Under

non-reducing conditions, no oligomerization of either the *h*E-LecEGF or the deglycosylated *h*E-LecEGF was observed, indicating that *h*E-LecEGF is a monomer, and coinciding with the published result that the soluble form of E-selectin is a monomer[336].

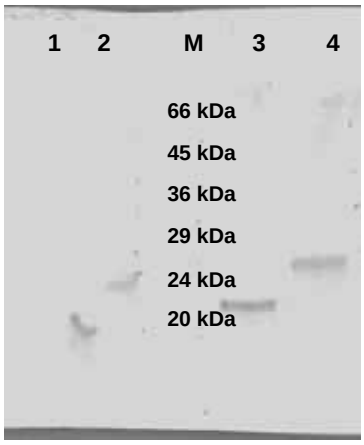


Figure 3.54. Western blotting analysis of the *h*E-LecEGF and the deglycosylated *h*E-LecEGF with the monoclonal anti-E-selectin antibody N18 under reducing and non-reducing conditions. Lane M: Low molecular weight marker; Lane 1: Deglycosylated *h*E-LecEGF under non-reducing conditions; Lane 2: *h*E-LecEGF under non-reducing conditions; Lane 3: Deglycosylated *h*E-LecEGF under reducing conditions; Lane 4: *h*E-LecEGF under reducing conditions.

3.3.4.4 Yield determination

The amount of the purified *h*E-LecEGF was quantified by Bradford micro-assay as described in section 2.3.7.4 480 μg/L and 762 μg/L of pure *h*E-LecEGF were obtained from T flasks production and roller bottles production respectively (Figure 3.55). It showed about 1.6 times higher of the protein produced in roller bottles than in T flasks due to the advantages of the non-static culture of roller bottles. Compared to the published result that ~ 800 μg/L of the crude *h*E-LecEGF (quantified by sandwich ELISA with mAb 1D6 and biotinylated mAb 3B7) was produced in fermentation before the purification[36], 762 μg/L of pure *h*E-LecEGF was obtained after the functional purification, indicating that a good *h*E-LecEGF stable expression subclone CHO-YJES1 was achieved.

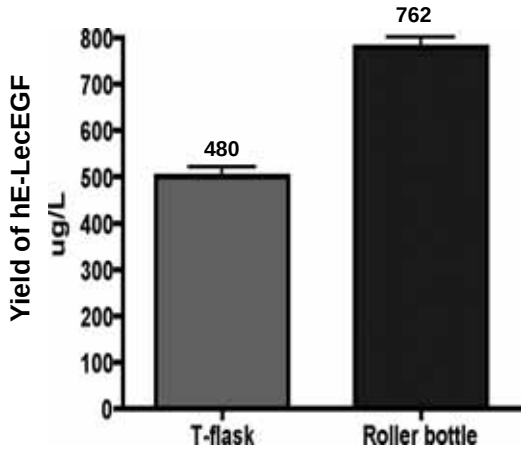


Figure 3.55. Yields of *h*E-LecEGF purified by the mAb 7A9 coupled column, quantified by Bradford micro-assay.

3.3.4.5 Peptide identification by mass spectrometry

The amino acid sequence of *h*E-LecEGF was identified by the ESI-MS-MS method. Reduced *h*E-LecEGF (2 μg) was separated by SDS-PAGE and stained with Simply Blue SafeStain (Invitrogen). After treatment with DTT, iodoacetamide and trypsin, the digested *h*E-LecEGF was analyzed by LC/MS/MS as described in section 2.3.7.5.

6 of 9 expected fragments were found except the fragments F1 (amino acids 1-22), F3 (amino acids 33-54) and F8 (amino acids 109-143) (Figure 3.56 and Table 3.5). ~ 50% of *h*E-LecEGF amino acids including the binding active sites (Glu80, Asn82, Asn105 and Asn106) were identified, indicating the purified protein was human E-LecEGF. The missing of the fragments F1 and F8 could be due to the N-glycosylation in these two fragments, since three potential N-glycosylation sites (Asn4, Asn124 and Asn139) predicted by NetNglyc1.0 server (see section 3.1.2.1) are present in these two fragments, the glycosylation on these fragments changed their molecular weights, leading to the failed identification of these fragments.

Table 3.5. *h*E-LecEGF fragments identified in ESI-MS-MS.

Fragment No.	M+H	Amino acids NO.
F2	1186.66	23-32
F4	2326.26	55-74
	2198.16	56-74
F5	1154.53	75-84
F6	1482.71	85-96
	1638.82	85-97
F7	1378.61	98-108
F9	923.43	144-152
	1495.69	144-157

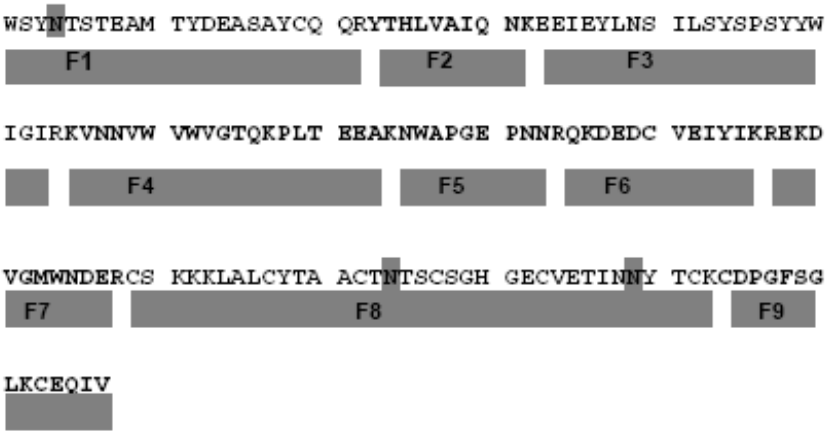
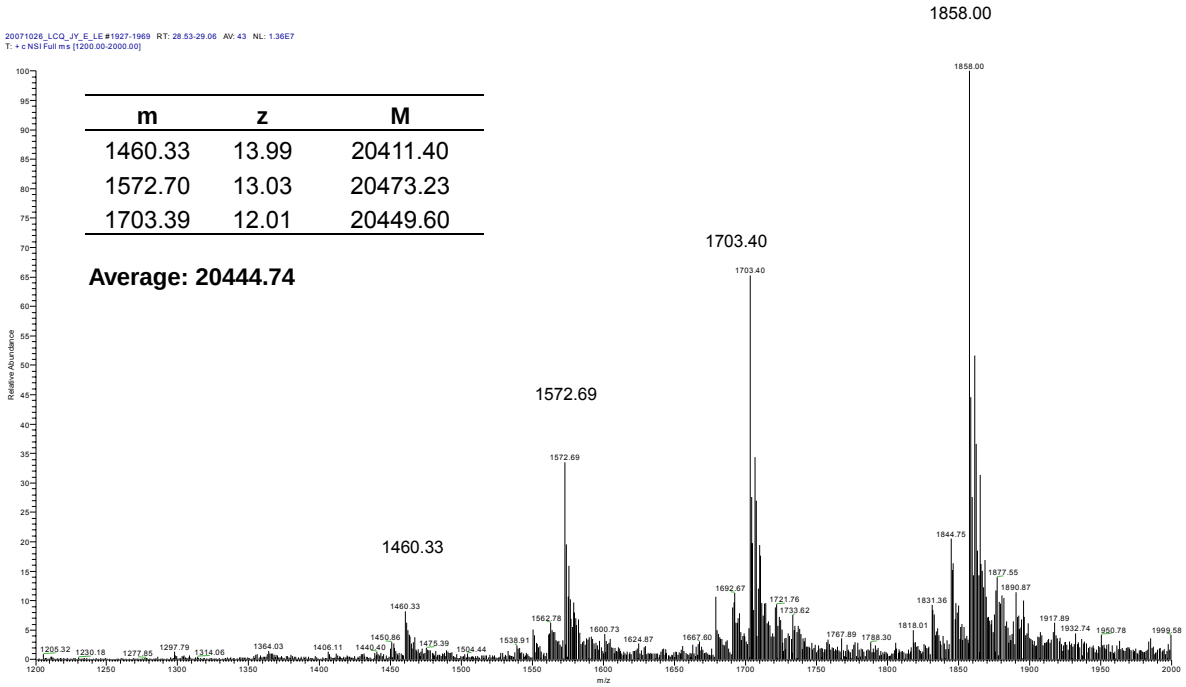


Figure 3.56. The found fragments (bold amino acids) of *h*E-LecEGF in peptide identification by ESI-MS-MS. N in grey is the potential glycosylation site.

3.3.4.6 Molecular weight determination by mass spectrometry

The molecular weight of *h*E-LecEGF was determined by ESI-MS. Purified *h*E-LecEGF (10 μg) were further prepared by RP-HPLC under the gradient elution condition to remove $CaCl_2$ in the protein buffer (see section 2.3.5.3). The peak with the retention time of 4.721 min in RP-HPLC chromatography was collected and lyophilized overnight before MS measurement.

The molecular weight of *h*E-LecEGF was measured by ESI-MS and determined as 20.444 kDa as described in section 2.3.7.5 (Figure 3.57). According to the predicted molecular weight of 18.1 kDa and the deglycosylation results in section 3.3.4.2, *h*E-LecEGF was N-glycosylated with 2.344 kDa of glycans. The three potential N-glycosylation sites predicted by NetNglyc1.0 server were Asn4, Asn124 and Asn139. Based on the glycosylation mode in CHO cells (Figure 1.10), the complete molecular weight of the glycan on one asparagine is 2.35 kDa, the glycan of 2.344 kDa on *h*E-LecEGF expressed in CHO cells indicated that the *h*E-LecEGF protein was partially glycosylated on one or two or all the three potential glycosylation sites, since the CHO cell does not maintain the complete glycosylation under the production conditions[259].



m	z	M
1460.33	13.99	20411.40
1572.70	13.03	20473.23
1703.39	12.01	20449.60

Average: 20444.74

Figure 3.57. ESI-MS spectrum of *h*E-LecEGF.

3.3.4.7 Activity determination

The activity of the *h*E-LecEGF and the deglycosylated *h*E-LecEGF was tested by western-blotting, ELISA, polymer assay and NMR methods, respectively.

(1) Western blotting analysis with the mAb 7A9

The activity of the deglycosylated *h*E-LecEGF (1.5 μg) was identified by western blotting with the mAb 7A9 under native conditions as described in section 2.3.7.6. The signal on the membrane (Figure 3.58) showed that the deglycosylated *h*E-LecEGF was recognized by the functional blocking anti-E-selectin antibody, indicating the deglycosylated *h*E-LecEGF was active and correctly folded.

Figure 3.58. Western blotting analysis of the deglycosylated *h*E-LecEGF with the mAb 7A9.

2) ELISA

ELISA of the *h*E-LecEGF protein (5 μg) and the deglycosylated *h*E-LecEGF (5 μg) was also performed with the mAb 7A9 to identify the activities of the protein (see section 2.3.7.6). The results showed that the *h*E-LecEGF and the deglycosylated *h*E-LecEGF were active (Figure 3.59).

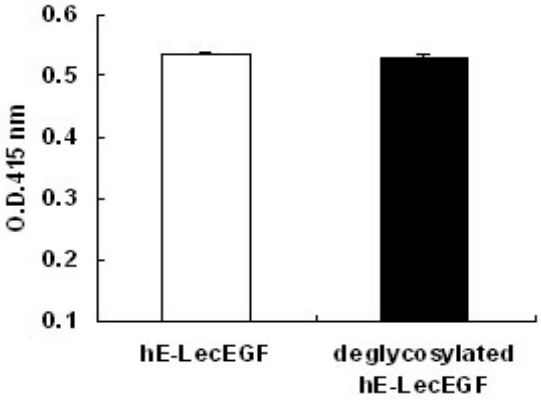


Figure 3.59. ELISA of the *h*E-LecEGF and the deglycosylated *h*E-LecEGF with the mAb 7A9.

(3) Polymer binding assay

Polymer binding assay[325] of *h*E-LecEGF with sLe^a-polymer-biotin-streptavidin-POD complex (sLe^a-polymer complex) was performed to identify the activity of *h*E-LecEGF (see section 2.3.7.6). Pure *h*E-LecEGF was directly coated onto the MaxiSorb 96-well ELISA plates (Nunc). The sLe^a-polymer complex was used to detect the binding of *h*E-LecEGF to its ligand sialyl Lewis a (sLe^a). After colour development with peroxidase substrate ABTS, the binding of sLe^a was analyzed by the software Prism 4.

Unexpected, the binding signal of the polymer assay was very low for detection even after the optimization with different concentrations of *h*E-LecEGF (5 μg/ml, 10 μg/ml and 20 μg/ml) and sLe^a polymer complex (0.1 μg/ml and 0.5 μg/ml), binding buffer (HAB20 buffer containing 20 mM $CaCl_2$) and prolonged developing time (10 min-20 min) (Figure 3.60).

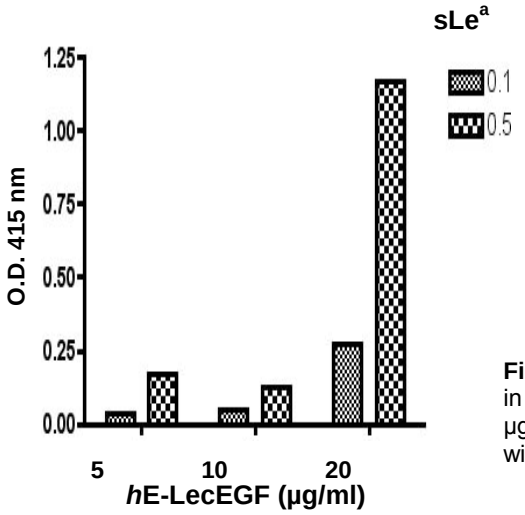


Figure 3.60. Optimization of the polymer binding assay in different concentrations of *h*E-LecEGF (5, 10 and 20 μg/ml) and sLe^a (0.1 and 0.5 μg/ml) in HAB20 buffer with a developing time of 15 min.

Compared with the positive control of *h*E-selectin/IgG[333] in the polymer assay, the binding signal of *h*E-LecEGF and LecEGFFlag (Z) (*h*E-LecEGF fused with a C-terminal flag tag)[324] are very low at concentrations of 5 μg/ml or 10 μg/ml (Figure 3.61). This might be due to the bad coating of the *h*E-LecEGF protein to MaxiSorb 96 well ELISA plate, as previously reported in the reference[36]. Bradford assay was used to check the coating of *h*E-LecEGF in ELISA plates. The weak signal from the *h*E-LecEGF protein immobilized in ELISA plates showed that *h*E-LecEGF can not be directly immobilized well onto the ELISA plates. Therefore, an immobilization strategy for the *h*E-LecEGF protein was developed and optimized for the binding assay (see section 3.4).

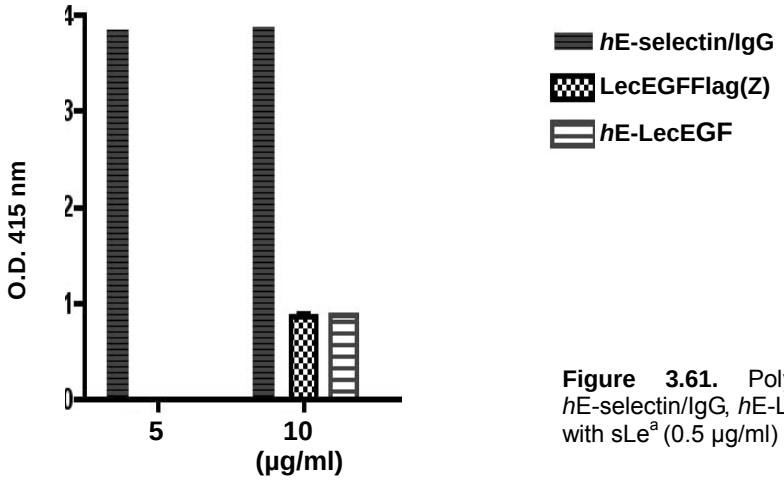


Figure 3.61. Polymer binding assay of *h*E-selectin/IgG, *h*E-LecEGF and LecEGFFlag(Z) with sLe^a (0.5 μg/ml) in HAB20 buffer.

(4) NMR assay

An STD NMR experiment and a T1rho experiment were performed to assess the binding of *h*E-LecEGF with its ligand CGP69669 (one antagonist of human E-selectin) as described in section 2.3.7.6. Pure *h*E-LecEGF (52 μM) in a 200 μl NMR buffer (25 mM Tris, 150 mM NaCl, 20 mM $CaCl_2$ in D_2O) was used. CGP69669 is a sLe^x mimic. It shows a 10-fold increase in binding affinity to *h*E-selectin than sLe^x[252]. Its structure is shown in Figure 3.62, the cyclohexandiol and S-cyclohexylactic acid in CGP69669 replacing the Glc*N*Ac and sialic acid in sLe^x (Figure 1.8a) respectively.

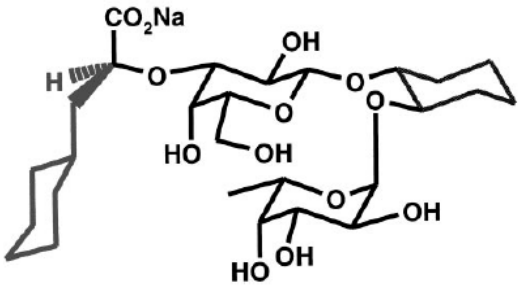


Figure 3.62. Structure of CGP69669[252].

- **STD NMR experiment**

A saturation transfer difference (STD) NMR experiment measures the intermolecular Overhauser transfer of magnetization and gives the information on the binding events and the binding epitopes of the ligands upon interaction with the protein. Due to the mechanism of the Overhauser process, the transfer is strongly distance-dependent and limited to internuclear distances of approximately 4 Angstroms or less. This enables a qualitative assessment of binding as evidenced by a signal from the ligand in the STD spectrum upon irradiation of the protein.

The STD result is shown in Figure 3.63. The binding signal of CGP69669 to *h*E-LecEGF is similar to that of CGP69669 to *h*E-selectin/IgG (positive control)[324], both of them showed the binding signal of 2-hydroxy group of Gal in CGP69669 to proteins in position of 3.6 – 4.0 ppm, whereas *h*E-LecEGF alone (negative control) did not show any signal. This indicates that CGP69669 binds to the *h*E-LecEGF protein in the same binding mode as *h*E-selectin/IgG. Compared with *h*E-selectin/IgG, the weaker signal of *h*E-LecEGF in the STD experiment could be due to the molecular weight of *h*E-LecEGF (20.444 kDa) which is 14 times smaller than *h*E-selectin/IgG (296 kDa).

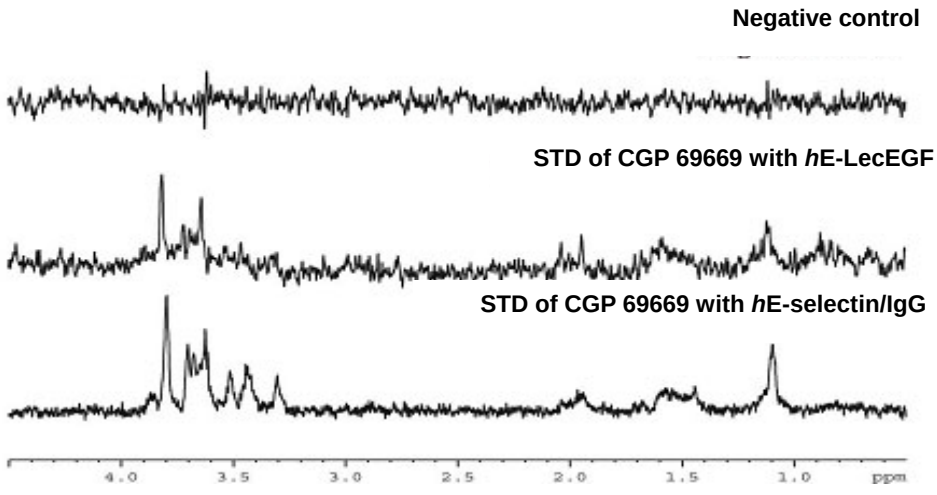


Figure 3.63. STD spectrum of CGP 69669 binding with *h*E-LecEGF or *h*E-selectin/IgG, the negative control is *h*E-LecEGF alone.

- **T1rho experiment**

The qualitative assay of T1rho (Transverse relaxation) NMR experiment was performed as a complementary diagnosis of the binding of *h*E-LecEGF (see section 2.3.7.6). Transverse relaxation determines the ligand binding because the rate of relaxation increases with molecular weight[341]. Hence, a ligand that binds to a large receptor displays a faster decay of magnetization (a greater rate of relaxation) than the free ligand. The obtained T1rho spectrum of CGP69669 displayed the increased relaxation of ligand CGP69669 in the presence of *h*E-LecEGF (Figure 3.64), indicating the binding of CP69699 to *h*E-LecEGF.

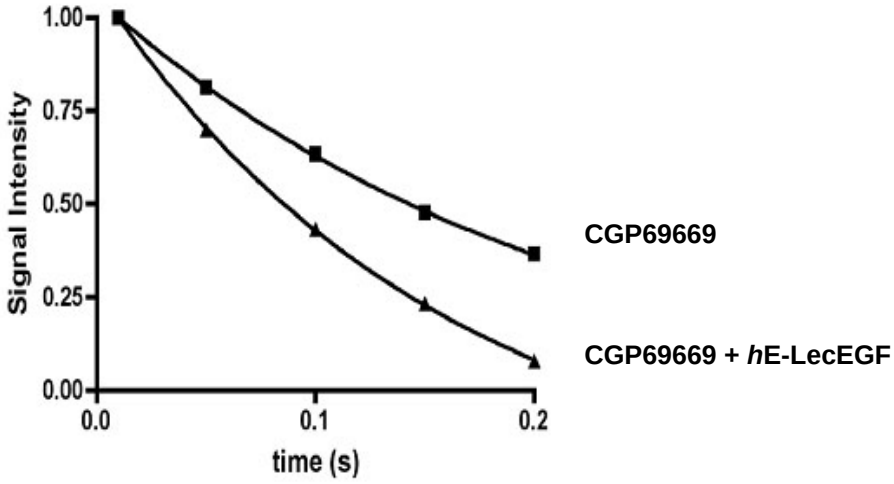


Figure 3.64. T1rho spectrum of CGP 69669 and CGP69669 with *h*E-LecEGF.

The qualitative assays of T1rho and STD NMR experiments both showed that CGP69669, a known antagonist of E-selectin, binds to the purified *h*E-LecEGF, indicating that the purified *h*E-LecEGF is active.

3.4 Capture-binding assay development

To date, the binding activity of *h*E-LecEGF has only been screened by cell-based assay[36]. The binding affinity of human E-selectin antagonists has only been evaluated with *h*E-selectin/IgG[325] or LecEGFFlag[324] *in vitro*. *h*E-selectin/IgG[325] and LecEGFFlag[324] contain the C-terminal *h*IgG1 tag and the C-terminal flag tag respectively. These tags may interfere with the binding activity of the protein.

In order to determine the binding affinity of *h*E-selectin antagonists to a monomeric, soluble and tag-free protein, *h*E-LecEGF was used to set up a cell-free capture-binding assay for the first time.

In previous investigations of *h*E-LecEGF (see section 3.3.4.7), the reason for the polymer assay failure has been the problem of immobilization of *h*E-LecEGF onto ELISA plates[36]. Therefore, a monoclonal anti-E-selectin antibody 1D6 [mAb 1D6, Roche (USA)] has been used to capture the *h*E-LecEGF in ELISA plates to evaluate the binding activity of *h*E-LecEGF in a cell-based assay[36]. Since mAb 1D6 is not available, a suitable anti-E-selectin antibody for the *h*E-LecEGF capturing was sourced. The ideal antibodies should recognize *h*E-LecEGF but with no or low interference in the binding activity of *h*E-LecEGF like the mAb 1D6.

A commercial monoclonal anti-E-selectin/anti-P-selectin antibody BBA1 (mAb BBA1) showed similar binding features to the lectin domain of *h*E-selectin like the mAb 1D6[25] (Table 3.6). Their binding affinities are sensitive to the mutagenesis of amino acids of Y12, Y18, Q20, R22, N39, Y44, (K96, R97, K99), (K111, K112, K113) in the lectin domain of human E-selectin (see Table 3.6), indicating the epitopes recognized by mAb 1D6 and BBA1 are these amino acids. According to the previous crystal structural reports[324], these amino acids were not involved in the binding of the protein. Hence, the mAb BBA1(R&D system) was chosen for immobilization of *h*E-LecEGF in ELISA plates.

Table 3.6. Summary of the binding of *h*E-selectin/IgG mutants with anti-E-selectin antibodies[25].

	mAb binding															
E selectin-IgG mutant	**BBA1**	**BBA2**	**ENA1**	**3B7**	**8 E4**	**7H5**	**9H9**	**1B3**	**11G5**	**1 E5**	**14G2**	**4D9**	**1D6**	**9A1**	**7 E10**	**sLe^x binding**
S2T N4H T5Y	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T7A A9N	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+	ND
E8A	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	
M10Y T11S Y12W	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K32A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S43A	+	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+
S45A	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+
P46A	+	+	+	+	±	+	+	+	+	+	+	+	+	+	+	±
S47A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±
S47D	+	+	+	+	±	-	+	+	+	+	+	+	+	+	+	-
Y48F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
K67A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K74A	+	+	+	+	+	+	-	-	-	+	±	-	-	+	+	+
R84A K86A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
R97A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
E98P VI01T	+	+	+	-	+	±	+	+	+	+	+	+	+	+	+	ND
K99A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±
D100A	+	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+
V101A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E107A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K111A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
K113A	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-
M10A	-	-	-	-	-	-	-	-	-	-	-	-	-	±	-	-
Y12A	-	-	-	-	-	-	-	-	-	-	-	-	-	±	-	-
E14A	not expressed															
Y44A	not expressed															
Y48A	+	+	±	±	±	±	±	±	±	±	±	±	±	+	±	ND
Y49A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND
E92A	+	+	±	±	±	-	±	±	±	±	±	±	±	+	+	ND
I93A	ND	ND	ND	-	-	-	-	-	-	-	-	-	-	±	±	ND
Y94A	ND	ND	ND	-	-	-	-	-	-	-	-	-	-	-	-	ND
I95A	ND	ND	ND	-	-	-	-	-	-	-	-	-	-	-	-	ND
K96A	-	-	-	-	-	-	-	-	-	-	-	-	-	±	±	-
E98A	ND	ND	ND	-	-	-	-	-	-	-	-	-	-	±	±	ND
M103A																-
E14I, A15S, SI6R, A17K	ND	ND	ND	-	-	-	-	-	-	-	-	-	-	-	-	ND
Y18A, Q20A, R22A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N39A, Y44A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
R54A, K55A, N57A	-	-	-	-	-	-	-	-	-	-	-	-	-	±	±	-
K96A, R97A, K99A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
K111S, K112A, K113A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+, Binding; +, partial binding; -, no binding.

3.4.1 Assay development

A cell-free capture-binding assay was developed based on the reported polymer assay[325] and cell-based binding assay[36], in order to evaluate the binding affinity of *h*E-selectin antagonists (see section 2.4.2). The anti-E-selectin/anti-P-selectin mAb BBA1 was first coated onto the MaxiSorb 96 well ELISA plates (Nunc) at 4°C, overnight. After blocking with 3% BSA, *h*E-LecEGF was captured by the mAb BBA1 during the incubation at 4°C, overnight. The binding activity of *h*E-LecEGF was then evaluated by the binding of sLe^a-polymer complex. This assay can also be used to determine the binding activity of *h*E-LecEGF.

A preliminary screening was performed to optimize the assay conditions with different concentrations of mAb BBA1 (3 µg/ml, 5 µg/ml and 10 µg/ml), *h*E-LecEGF (1 µg/ml, 3 µg/ml, 5 µg/ml and 10 µg/ml) and sLe^a-polymer (0.1 µg/ml, 0.3 µg/ml and 0.5 µg/ml) as described in section 2.4.3. The strong and stable signal was obtained with 3 µg/ml of BBA, 1 µg/ml of *h*E-LecEGF and 0.1 µg/ml of sLe^a-polymer in HAB20 buffer with the developing time of 5 min (Figure 3.65). This assay condition was used for the following capture-binding assay and the competitive binding assay of *h*E-LecEGF.

For qualitative determination of the binding activity of *h*E-LecEGF, 233 µg of *h*E-LecEGF was needed in NMR assay, whereas 0.5 µg of *h*E-LecEGF was enough to perform the capture-binding assay: much less protein was consumed by the capture-binding assay.

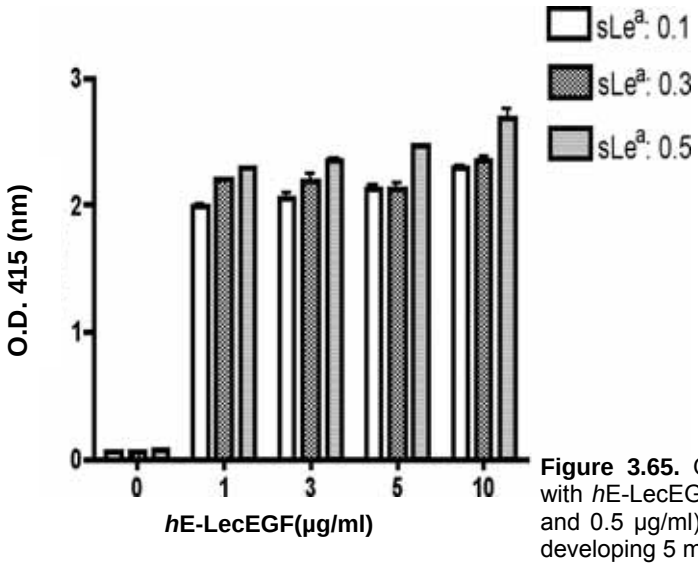


Figure 3.65. Optimization of the capture-binding assay with *h*E-LecEGF (1,3, 5 and 10 µg/ml) and sLe^a (0.1, 0.3 and 0.5 µg/ml) with 3 µg/ml of BBA1in HAB20 buffer for developing 5 min.

3.4.2 EC_{50} determination of sLe^a-polymer

The EC_{50} of the sLe^a polymer was measured by the capture-binding assay with the sLe^a polymer in the concentration range of 0.3 ng/ml - 300 ng/ml (see section 2.4.4). The binding curve and the value of EC_{50} of sLe^a polymer were determined with the Prism4 software by using a non-linear regression fit (Figure 3.66). The EC_{50} of the sLe^a polymer to *h*E-LecEGF was determined as 21.65 ng/ml. Compared with 48.28 ng/ml of EC_{50} of the sLe^a polymer to *h*E-selectin/IgG measured by the polymer assay (done by Katrin Lemme, IMP), sLe^a polymer showed stronger binding affinity to *h*E-LecEGF than to *h*E-selectin/IgG. 1.5 µg of *h*E-LecEGF was required for determination of the EC_{50} value of sLe^a polymer to *h*E-LecEGF.

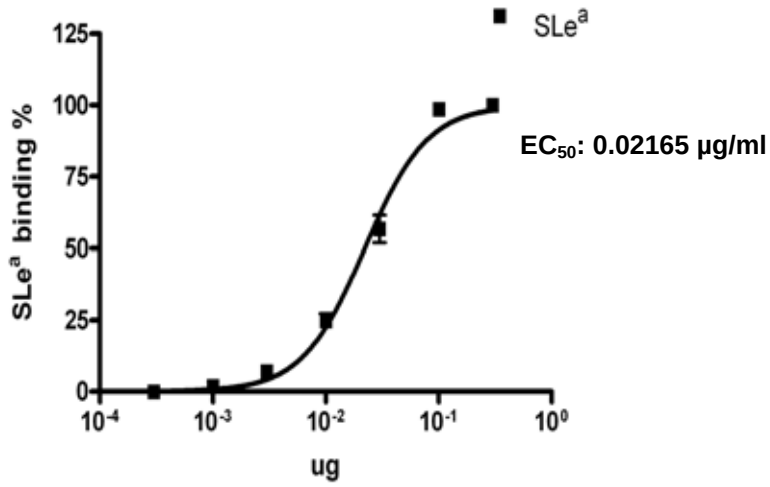


Figure 3.66. The binding curve and EC_{50} determination of sLe^a polymer to *h*E-LecEGF, analyzed by the Prism4 software.

3.4.3 Competitive capture-binding assay

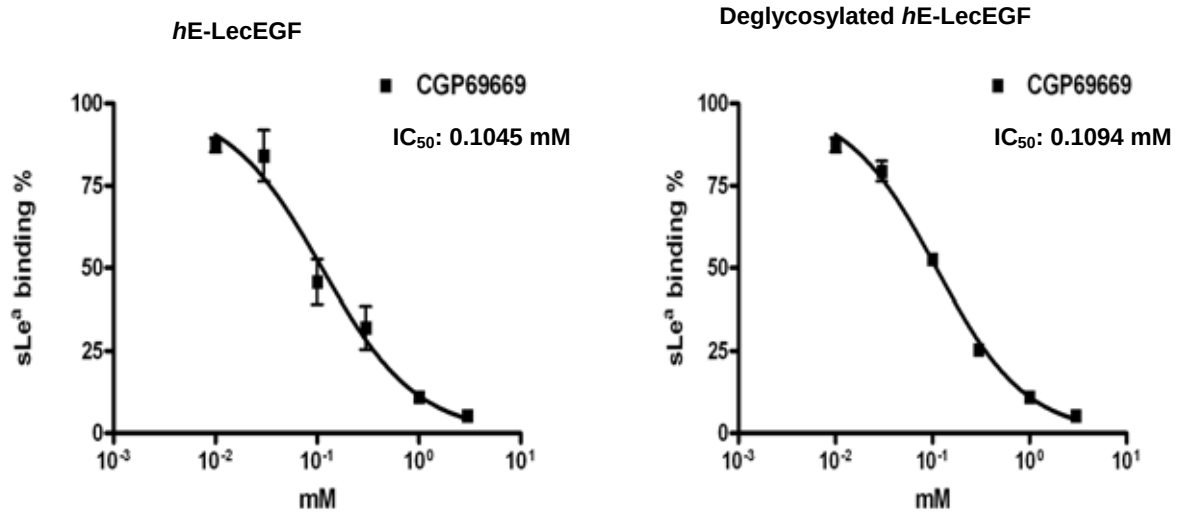
In order to evaluate potent *h*E-selectin antagonists, the competitive capture-binding assay was developed based on the capture-binding assay by adding serial concentrations of *h*E-selectin ligand with 0.1 µg/ml of sLe^a simultaneously (see section 2.4.5). The IC_{50} of the antagonists was measured in this assay and calculated by the Prism4 software.

3.4.3.1 IC_{50} of CGP69669 to *h*E-LecEGF and deglycosylated *h*E-LecEGF

CGP69669 is an antagonists of *h*E-selectin[252] with an IC_{50} of 110 µM to *h*E-selectin/IgG in polymer assay[342,343].

In order to evaluate the binding activities of *h*E-LecEGF and deglycosylated *h*E-LecEGF, the IC_{50} of CGP69669 to these proteins were determined by the competitive capture-binding assay, (Figure 3.67). CGP69669 showed the IC_{50} of 109.4 μM for the deglycosylated *h*E-LecEGF protein and 104.5 μM for the native protein, indicating that the deglycosylated *h*E-LecEGF was as active as the *h*E-LecEGF and the removal of glycans on *h*E-LecEGF did not affect its binding activity as reported in the previous study[30].

In addition, the IC_{50} of 104.5 μM of CGP69669 to *h*E-LecEGF is similar to the published value of IC_{50} of 110 μM of CGP69669 to *h*E-selectin/IgG[342,343], indicating the capture binding assay can correctly evaluate the binding affinity of human E-selectin antagonists.



a. b.

Figure 3.67. Competition binding curves and IC_{50} determination of ligand CGP69669 to *h*E-LecEGF (a) and to deglycosylated *h*E-LecEGF (b).

3.4.3.2 IC_{50} of six potent antagonists to *h*E-LecEGF

The binding affinities of six potent antagonists of human E-selectin (CGP69669, DS05-48, BW-408-0, DS05-65, DS05-102 and BW-186-1) were measured twice in triplicates in the competitive capture-binding assay. The competition binding curves (Figure 3.68, Figure 3.69 and Figure 3.70) and IC_{50} values of each ligands to *h*E-LecEGF were determined by the Prism 4 software. A parallel rightward shifts of dose–dependent curves of the ligands of DS05-48, BW-408-0, DS05-65 and CGP69669 was observed, indicating the rank order of the binding affinity is DS05-48 > BW-408-0 > DS05-65 > CGP69669 (Figure 3.68).

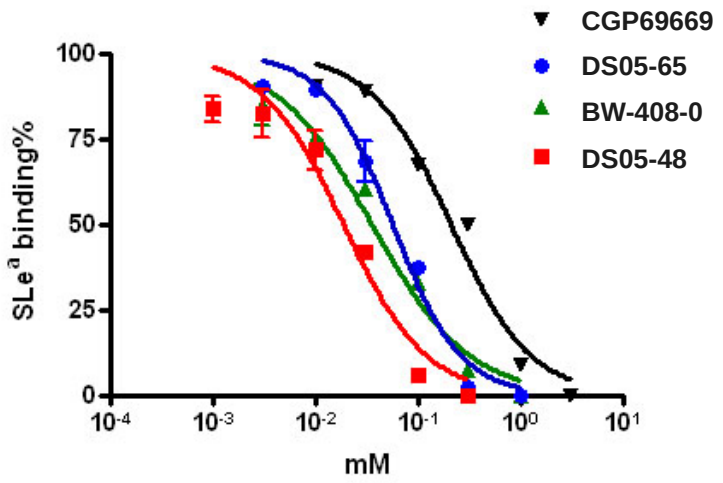


Figure 3.68. Competition binding curves of ligands CGP69669, DS05-65, BW-408-0 and DS05-48 to *h*E-LecEGF.

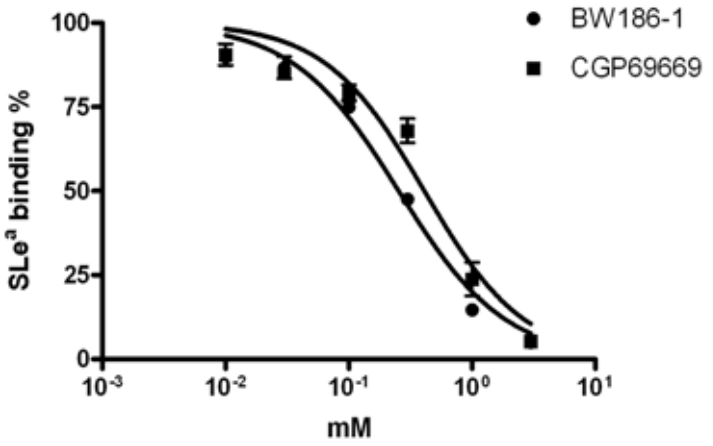


Figure 3.69. Competition binding curves of ligands BW186-1 and CGP69669 to *h*E-LecEGF.

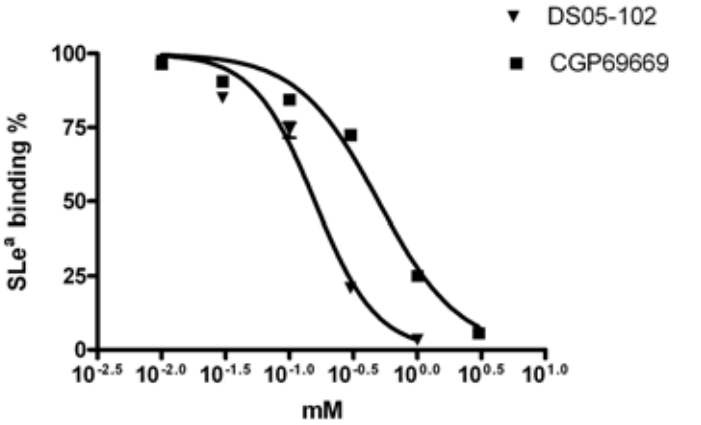


Figure 3.70. Competition binding curves of ligands DS05-102 and CGP69669 to *h*E-LecEGF.

By taking the IC_{50} of CGP69669 as a reference (IC_{50}: 150 μM, measured in the present assay), Table 3.7 shows the rIC_{50} value of each ligand determined by the capture-binding assay and the calculated results from the published data[342]. The values of KD and rKD of three ligands (CGP69669, BW-408-0 and DS05-65) obtained from surface plasmon resonance assay (Biacore) are also shown in Table 3.7 (see section 2.4.6). The value of KD evaluates the equilibrium binding of the ligands to *h*E-LecEGF in a steady state. The rKD values of these ligands were calculated using the ligand of CGP69669 as a reference.

Table 3.7. The rIC50, KD and rKD values of *h*E-selectin antagonists.

Ligands	Mw	rIC_{50} (Calculated from published results)	rIC_{50} (Measured by capture-binding assay)	KD (μM)	rKD
CGP69669	600.6282	1	1	58	1
DS05-48	768.8416	0.1	0.085		
BW-408-0	592.6729	0.2/0.1625	0.158	7.76	0.134
DS05-65	722.8168	0.4	0.28	5.3	0.09
DS05-102	760.8406		0.32*		
BW-186-1	819.8216		0.55		

* Not accurate, due to the low solubility in the assay buffer.

In the capture binding assay, the rIC_{50} values of 0.158 and 0.085 for BW-408-0 and DS 05-48 respectively, are in close agreement with the published results of 0.1625/0.2 and 0.1 (Table 3.7)[342]. For BW-408-0, the rIC_{50} of 0.158 measured by the capture-binding assay is similar to the rKD of 0.134 measured by Biacore (Table 3.7). Compared with the rIC_{50} of 0.4 for DS05-65 calculated from published results[342], the rIC_{50} of 0.28 for the same ligand measured by the capture-binding assay is closer to the rKD of 0.09 measured by Biacore. The difference between the values obtained from the capture-binding assay and Biacore could be due to the fact that Biacore is a dynamic assay, whereas the capture-binding assay is a static assay.

All these obtained results demonstrated that the developed competitive capture-binding assay can correctly evaluate the binding affinity of potent *h*E-selectin antagonists. In summary, compared with the unstable polymer assay[324], the capture binding assay is accurate, sensitive and reproducible.

3.5 Crystallization of *h*E-LecEGF

Crystallization conditions of *h*E-LecEGF were pre-screened with a sitting-drop vapour-diffusion method as described in section 2.5.2. Pure protein was concentrated to the final concentration of 9 mg/ml in a 60 μl buffer (20 mM Tris, 2 mM $CaCl_2$, pH 7.4) by ultra centrifugation. The droplet for crystallization, composed of 100 nl of protein plus 100 nl of reservoir solution, was prepared by an automated dispensing instrument. The crystallization of *h*E-LecEGF was performed on 96 well crystallization plates (Greiner) with Hampton crystallization screening I and II kits at 4°C and 20°C for 3 basic screening blocks. Until now, only the precipitation (Figure 3.71) and a salt crystal identified by X-ray shooting (Figure 3.72) have been obtained. The glycans on *h*E-LecEGF could increase the solubility of protein in the crystallization buffer, causing greater difficulties in obtaining the crystal. Hence, more pure protein of the deglycosylated *h*E-LecEGF needs to be produced in order to reveal the appropriate crystallization conditions of the protein co-crystallized with its ligands for further structural studies.

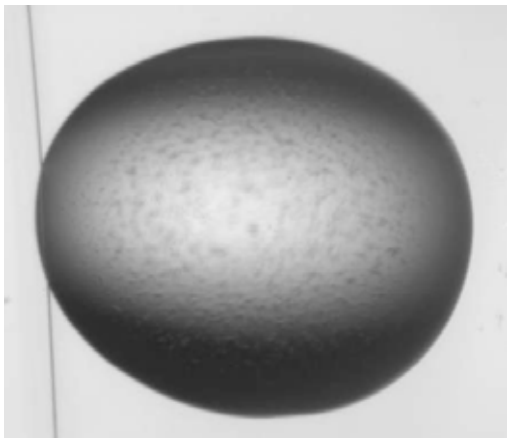


Figure 3.71. Precipitants formed in the crystal drop of *h*E-LecEGF.

Figure 3.72. A salt crystal formed in the crystal drop of *h*E-LecEGF, identified by X-ray shooting.

4. Conclusion and Outlook

Human E-selectin is a C-type lectin. It is transiently expressed on endothelial cells by induction of cytokines. It contains an N-terminal C-type lectin domain, an EGF-like domain, six CR domains, a transmembrane region and a cytoplasmic region. The key role of E-selectin is to mediate the initial rolling and adhering of leukocytes in the leukocyte recruitment in inflammation and metastasis of some cancer cells. It is fundamentally involved in many physiological and pathological processes. Hence, E-selectin is an attractive target for developing anti-inflammation drugs. The lectin and EGF-like domains of *h*E-selectin (*h*E-LecEGF) were identified as the minimum functional unit. Structural studies of *h*E-LecEGF or *h*E-selectin/IgG complexed with its natural ligand sLe^x by crystallization or NMR were reported and utilized as the structural basis for novel drug design. Many potent antagonists with better binding affinity than sLe^x were reported. However, the crystal structure of potent antagonist (e.g. CGP69669) with the *h*E-LecEGF protein remains unknown. With such a structure, a better understanding of the interaction between the protein and the ligand would facilitate the drug design. To obtain such structural information and develop more potent antagonists of *h*E-selectin are challenging tasks. A sufficient amount of the pure, active and characterized *h*E-LecEGF protein is needed to meet the demands of the protein for structure determination. A cell-free binding assay with the tag-free *h*E-LecEGF protein is also required for evaluation of the potent antagonists *in vitro.*

In this thesis, *h*E-LecEGF was first cloned and transiently expressed in the insect cell expression system. The expression plasmid pFastBacYJSE was constructed for secretion of *h*E-LecEGF with an N-terminal flag tag (DYKDDDDK). The tag can be completely removed by the protease enterokinase. The *h*E-LecEGF protein was transiently expressed in both Sf9 and High five™ insect cells in a suspension culture. Pure protein was obtained using optimized anti-flag affinity chromatography with addition of 0.5% Triton X-100 in the washing buffer. The yield of purified *h*E-LecEGF showed that more protein was produced in High five™ cells (5.82 mg/L) than in Sf9 cells (4.99 mg/L). On SDS-PAGE, the purified *h*E-LecEGF displayed four main bands of a glycosylated type at a molecular weight of 24-29 kDa, as identified by western-blotting analysis with the anti-flag antibody. The positive results of ELISA and western-blotting analysis with the functional blocking anti-E-selectin antibody 7A9 (mAb 7A9) indicated that the purified *h*E-LecEGF protein is active. However, the homogeneous deglycosylated *h*E-LecEGF protein was not obtained by deglycosylation with PNGase F and N-glycosidase A either separately or when combined under native and denaturing conditions. *h*E-LecEGF expressed in insect cells showed resistance to these deglycosidases. This could

be due to the restrictions of these deglycosidases[278,279,285].

*h*E-LecEGF was then cloned and stably expressed in mammalian cells CHO K1 to obtain the homogeneous protein. Two expression plasmids pYJE and pYJEG were constructed for expression of *h*E-LecEGF without a tag or with a C-terminal human IgG1 tag. An enterokinase cleavage site (DDDDK) was introduced in the plasmid pYJEG for further removal of the tag. 5 high and stable expression subclones from a single cell, CHO-YJES1-5 and CHO-YJEGS1-5, were obtained by selection with geneticin and evaluation with AP-ELISA. The CHO-YJES1 subclone was used for production of the *h*E-LecEGF protein in roller bottles and T-flasks due to the higher expression and easier purification. Pure *h*E-LecEGF protein was obtained by a one-step functional purification using the mAb 7A9 coupled column, indicating mAb 7A9 produced by hybridoma cell, purified on a Protein A column and coupled to cyanogen bromide activated sepharose 4B matrix, can be used for functional purification of the *h*E-LecEGF protein. 762 μg/L purified *h*E-LecEGF protein were obtained from production in roller bottles and 480 μg/L in T-flasks. Compared with the published result[36], a good *h*E-LecEGF expressing stable subclone CHO-YJES1 was achieved. The purified *h*E-LecEGF protein was further characterized by MS, NMR and immunoassay methods. It was a glycosylated monomer as judged by its migration profile in reducing, non-reducing and native conditions. By ESI-MS-MS, the molecular weight of *h*E-LecEGF was determined as 20.444 kDa and ~ 50% of its amino acid sequence (including the calcium binding sites) was identified. The binding activity of *h*E-LecEGF was confirmed by NMR (T1rho NMR and STD NMR), western-blotting, ELISA and the capture-binding assay. Pure and homogeneous deglycosylated *h*E-LecEGF protein was obtained by deglycosylation with PNGase F under native condition and additional purification on a sepharose Q matrix. The binding activity of the deglycosylated *h*E-LecEGF protein was verified by western-blotting, ELISA and the capture-binding assay. The results showed that the deglycosylated *h*E-LecEGF was as active as the native *h*E-LecEGF.

A novel capture-binding assay was developed with the tag-free *h*E-LecEGF protein to evaluate the binding affinity of *h*E-selectin antagonists. Six known ligands were measured and the rIC_{50} of each ligand was determined. The obtained results were in close agreement with the published results[342]. Compared to the previous unstable polymer assay with *h*E-selectin/IgG[324], the capture-binding assay with *h*E-LecEGF is accurate, sensitive and reproducible. In addition, an anti-E-selectin/P-selectin antibody BBA1 used in the assay solved the immobilization problem of the *h*E-LecEGF protein onto ELISA plates. Therefore, we conclude that the capture-binding assay with the *h*E-LecEGF protein can correctly evaluate the binding affinity of E-selectin antagonists. It is a static, cell-free and reliable assay.

In outlook, the process of preparation of pure, active and homogeneous deglycosylated *h*E-LecEGF protein from the initial cloning to the final characterization was achieved in this thesis. Therefore, more pure and active deglycosylated *h*E-LecEGF can be prepared in the future by large-scale production and purification by the same procedure for further structural study, such as co-crystallization of the leading ligands (e.g. CGP69669) with the protein, to elucidate the exact binding mode of the leading compound and facilitate the design of novel potent antagonists of human E-selectin.

The novel capture-binding assay with the tag-free *h*E-LecEGF protein was developed in this thesis. It can correctly determine the binding affinity of *h*E-selectin antagonists and could, therefore, serve as an experimental tool to assist the design and synthesis of further antagonists. In addition, the problem of immobilization of truncated *h*E-LecEGF protein onto the ELISA plates was solved by using the BBA1 antibody to capture the *h*E-LecEGF protein in the assay. This immobilization method could be used for immobilization of the *h*E-LecEGF protein in surface plasmon resonance (Biacore) assay or other assays.

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Publications and Awards

[1] **J. Yu**, X.M. Yu, J.H. Liu "A thermostable manganese-containing superoxide dismutase from pathogen Chlamydia pneumoniae." *FEBS Lett.* **2004**, *562*, 22-26.

[2] F. Wu, **J.Yu**, R.Li "Novel biomimetic affinity ligands for human tissue plasminogen activator. "*Biochem Biophys Res Commun*. **2007**, *355*, 673-678.

[3] F. Wu, **J. Yu**, Gehring H. "Inhibitory and structural studies of novel coenzyme-substrate analogs of human histidine decarboxylase. " *FASEB J.* **2007**, Epub ahead of print.

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